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UNIVERSITY OF ALBERTA

PHYTOCHEMICAL INVESTIGATION OF THERMOPSIS RHOMBIFOLIA

BY

RICHARD KIT MING LAM



A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

IN

PHARMACEUTICAL SCIENCES (MEDICINAL CHEMISTRY)
FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

SPRING, 1995



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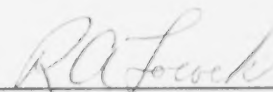
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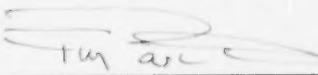
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled PHYTOCHEMICAL INVESTIGATION OF THERMOPSIS RHOMBIFOLIA in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in PHARMACEUTICAL SCIENCES (MEDICINAL CHEMISTRY).

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TO IBY AND PARENTS

ABSTRACT

Thermopsis rhombifolia, a perennial plant of the Leguminosae family, can be found in Alberta about 100 km south-east of Edmonton. This plant has been reported to be potentially poisonous to cattle and humans. The active poisonous constituents are suspected to be lupine-type alkaloids which occur particularly in several Leguminosae genera e.g. Thermopsis lupinoides.

The present phytochemical investigation of the basic fraction of the aerial part of this plant revealed five distinct alkaloids. Three of these alkaloids have been isolated and characterized: (-)-thermopsine, (-)-N-methyl-cytisine (caulophylline) and (-)-cytisine. The structures have been confirmed by spectroscopic methods and by direct comparison with a reference sample of (-)-cytisine and with the cited literature data. The structures of the other two alkaloids are not completely known but they are tentatively suggested to be (-)-rhombifoline and rhombinine which possess an 2-pyridone ring.

A simple and effective extraction and isolation technique has been developed for the determination of the alkaloidal constituents in this plant. The separation of the naturally occurring lupine-type alkaloids was performed by normal phase adsorption chromatography on silicic acid mixed with Celite®. The mobile phase was a mixture of dichloromethane, acetone, methanol and ammonia solution.

A high performance liquid chromatographic method was devised for the quantitative analysis of the lupine-type alkaloids. This analysis was performed by reversed-phase liquid chromatography on octadecylsilica. The mobile phase was a buffered aqueous solution containing acetonitrile and elution of alkaloids was monitored by UV absorption at 310 nm. The quantification of the lupine-type alkaloids was carried out by an external standard method. The variations in alkaloid content during flowering and seed-growth periods were examined.

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PART ONE

INTRODUCTION

The history of alkaloids is almost as old as civilization. Humankind has used drugs containing alkaloids in potions, medicines, teas, poultices, and poisons for 4000 years. No attempts were made to isolate any of the therapeutically active ingredients from the crude drugs until the early nineteenth century [1].

The first crude drug containing alkaloids to be investigated chemically was opium, the dried latex of the poppy Papaver somniferum. Opium had been used for centuries in popular medicine, and both its analgesic and narcotic properties were well known. In 1803, Derosne isolated a semipure alkaloid from opium and named it narcotine. Further examination of opium by Serturmer [2] in 1805 led to the isolation of the alkaloid morphine.

Alkaloids are the oldest drugs. Since the times of Hippocrates, extracts of plants have been known to serve as medicine for treatment of a number of diseases. Later, many of the active principles of plant extracts were identified as alkaloids. Even with the dramatic progress of organic chemistry which has resulted in an enormous production of synthetic drugs, some of the most powerful remedies are still of plant origin. Whereas organic synthesis of complicated molecules is often extremely costly, plants produce them with apparent ease and at little cost; so plants will remain important sources of some medicinal substances.

The isolation of lupine alkaloids from the Thermopsis rhombifolia was done by Manske and Marion [3] about 50 years ago. Structural investigation and quantification of the alkaloids present in this species, by modern spectroscopic methods, have not been reported. This plant is a native herb in Manitoba, Saskatchewan, Alberta and British Columbia; it would be interesting to have a deeper understanding of its alkaloidal characteristics.

PART TWO

LITERATURE SURVEY

A. Characterization of alkaloids

The first alkaloid isolated was coniine in 1827; this alkaloid was successfully synthesized in 1886 by Ladenburg. In many cases it took more than 100 years of intensive chemical research for the structure of alkaloids isolated to become known and be accepted in the field of organic chemistry. Nicotine discovered in 1828 was not synthesized until 1904, quinine isolated in 1820 was synthesized in 1944, morphine isolated in 1806 was synthesized in 1952, and strychnine isolated in 1818 was synthesized in 1954 [4-7].

A new era of alkaloid research began in the 1950's and the methods of alkaloid isolation became increasingly sophisticated. New physico-chemical procedures were introduced into the chemistry of natural products, resulting in a rapid decrease in the time between the isolation and structural determination of alkaloids. Since then the number of known alkaloids has greatly increased, as illustrated in Table 1.

Table 1. History of the discovery of alkaloids.

Year	Landmarks : the studies of alkaloids
1900	Pictet [8] reported about 100 alkaloids, whose chemical structures were all more or less unknown.
1908	Euler [9] described about 100 alkaloids, including about 30 with unknown chemical structure.
1922	Wolffenstein [10] reported about 150 alkaloids at least partially known and about 100 chemically unknown or almost unknown alkaloids.
1960	Boit [11] described about 2000 alkaloids, including about 600 with unknown chemical structure.
1970	Raffauf [12] listed about 4000 alkaloids.
1978	Waller and Nowacki [1] estimated the number of known alkaloids to be about 6000, 800 of which had been discovered in the two preceding decades.

Today about 7000 more or less well-characterized alkaloids may be known. Alkaloids have been detected in more than 100 plant families, but only about 4 % of all plant species have been investigated for alkaloids. Therefore the screening and characterization of alkaloids in plants offer a wide field for scientists [4].

B. Lupine alkaloids

The most important alkaloid-containing plant families are the Amaryllidaceae, Apocynaceae, Compositae, Lauraceae, Leguminosae, Liliaceae, Loganiaceae, Menispermaceae, Papaveraceae, Ranunculaceae, Rubiaceae, Rutaceae, and Solanaceae [13].

In a given genus, plants will often yield the same or structurally related alkaloids, and even several different genera within a family may contain similar alkaloids.

The alkaloids of lupine (Lupinus spp.) comprise about 30 different compounds with structural resemblances and apparent biochemical interrelationships. These compounds, often called "lupine alkaloids", are well known in related Leguminosae genera such as Baptisia, Cytisus, and Genista and are also found sporadically in other plant families. All contain one or two quinolizidine ring systems (Figure 1) and may also be called quinolizidine alkaloids. It has been reported that lupine alkaloids are synthesized and accumulated in the aerial parts of the plant [14].

Some major structural types of lupine alkaloids are shown in Figure 2 [15-23].

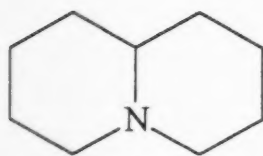
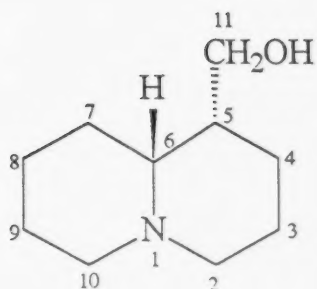
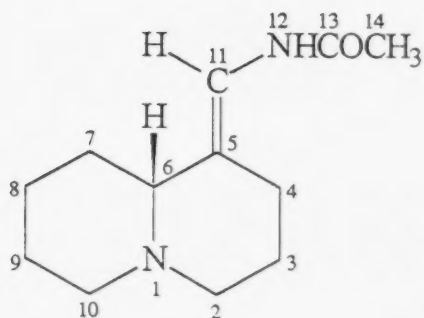


Figure 1. Quinolizidine ring system.

a. Lupinine type



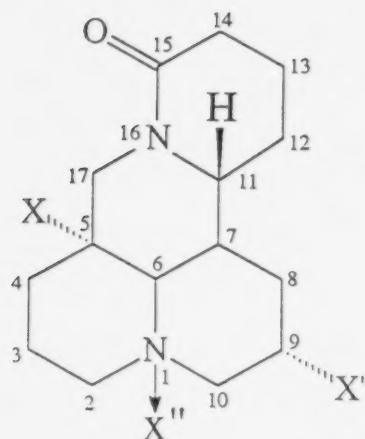
(I) (-)-lupinine



(II) (-)-lusitanine

Figure 2. Natural Lupine alkaloids.

b. Matrine type

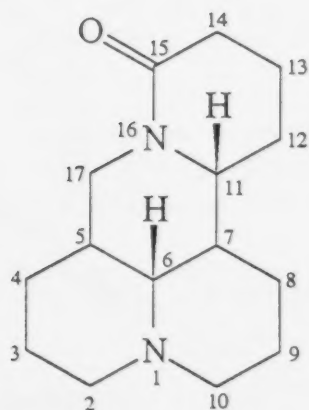


(IIIa) (+)-matrine : $X=H$, $X'=H$, $X''=\text{lone pair}$

(IIIb) (+)-sophoranol : $X=OH$, $X'=H$, $X''=\text{lone pair}$

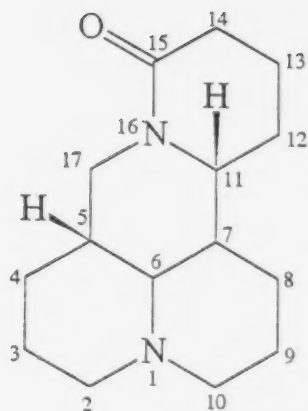
(IIIc) (+)-5 α ,9 α -dihydroxymatrine : $X=OH$, $X'=OH$,
 $X''=\text{lone pair}$

(IIId) (+)-matrine N-oxide : $X=H$, $X'=H$, $X''=O$



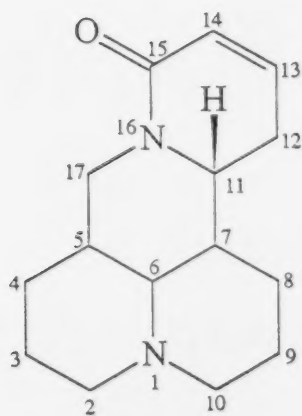
(IV) (+)-allomatrine

Figure 2. (continued)



(Va) (-)-sophoridine

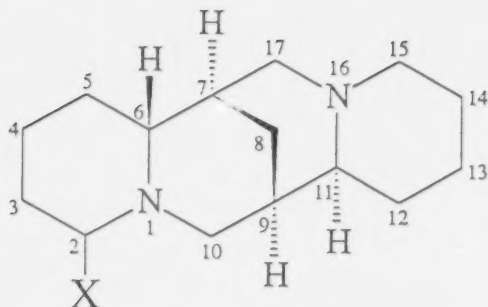
(Vb) (-)-sophoridine N-oxide : O at N-1



(VI) (-)-sophocarpine

Figure 2. (continued)

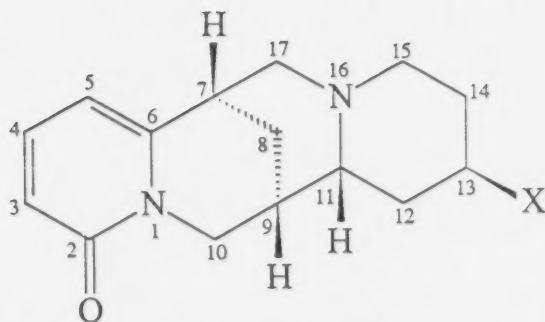
c. Sparteine type



(VIIa) (-)-sparteine : $X=H_2$

(VIIb) (+)-lupanine : $X=O$

d. Anagyrine type

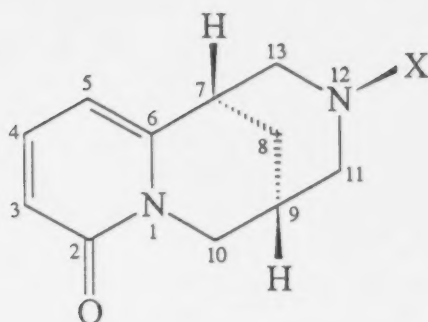


(VIIIa) (-)-anagyrine : $X=H$

(VIIIb) (-)-baptifoline : $X=OH$

Figure 2. (continued)

e. Cytisine type



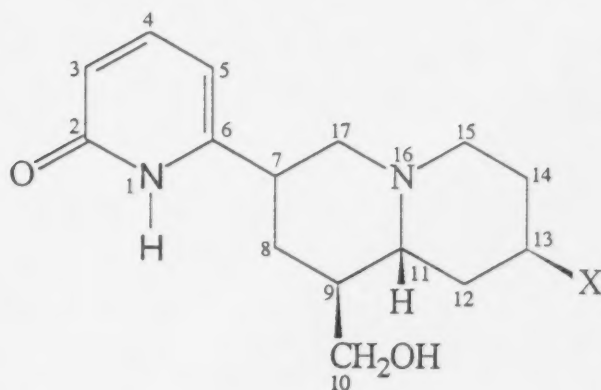
(IXa) (-)-cytisine : $X=H$

(IXb) (-)-N-methylcytisine : $X=CH_3$

(IXc) (-)-12-cytisineacetic acid : $X=CH_2COOH$

(IXd) (-)-methyl 12-cytisineacetate : $X=CH_2COOCH_3$

f. Mamanine type

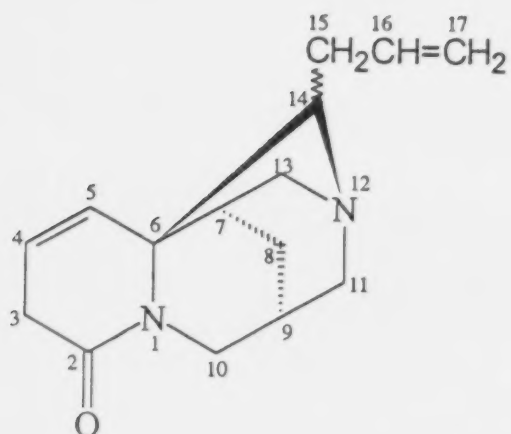


(Xa) (+)-mamanine : $X=H$

(Xb) (+)-13 β -hydroxymamanine : $X=OH$

Figure 2. (continued)

g. Tsukushinamine type



(XI) (-)-tsukushinamine A

Figure 2. (continued)

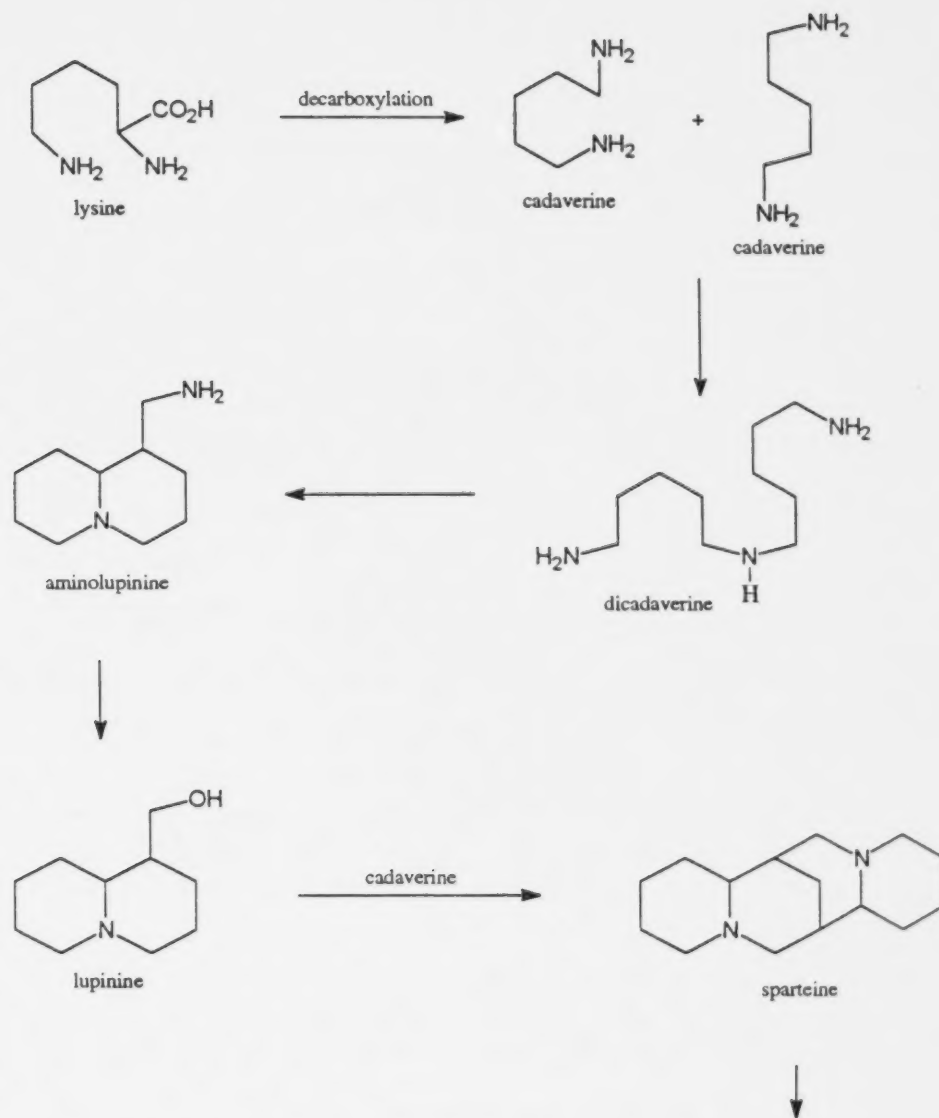
Okuda et al. [24] have pointed out that customarily a series of lupine alkaloids with the same configuration occur together in the same plant. In 1984, Ohmiya et al. [25] found that alkaloids having the opposite optical rotation occur together in Thermopsis lupinoides. But in 1989, Saito et al. [26] showed, by tissue culture of Thermopsis lupinoides, that the production of (+)-lupanine (VIIb) is related to the greening of the tissue but that of the enantiomeric alkaloids, e.g. (-)-lupanine, (-)-anagyrene, (-)-baptifoline, (-)-cytisine, (-)-N-methylcytisine, and (-)-N-formylcytisine, are not related to greening. There is no evidence to confirm that Thermopsis lupinoides synthesizes alkaloids of opposite absolute configuration.

The presence of alkaloids in the lupines may account for the fact that livestock avoid feeding on lupines if other forage is available [27].

C. Biochemistry of the lupine alkaloids

Lupine alkaloids particularly occur in different families of Leguminosae. The simplest alkaloid of this group is lupinine. Moreover, different tetracyclic derivatives such as sparteine and lupanine, as well as higher oxidized derivatives, and skeleton variants such as matrine are known.

Quinolizidine skeletons of all the major lupine alkaloids are built up from two or three C_5 units derived from lysine via its decarboxylation product cadaverine [28] (see Figure 3).



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Figure 3. Biogenetic relationships of lupine and related alkaloids.

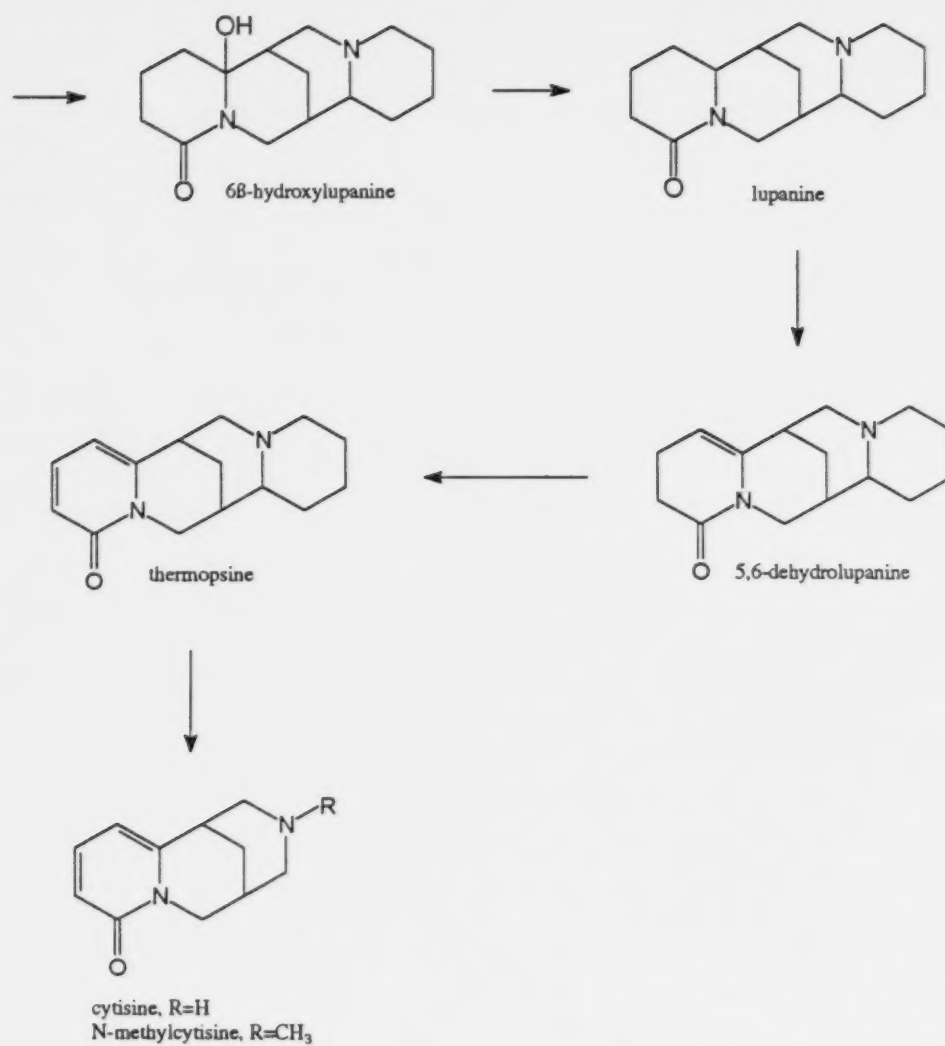


Figure 3. (continued)

D. Thermopsis rhombifolia

Thermopsis genus has its closest affinity to Baptisia, but the leaves do not blacken upon drying. The species are commonly called golden peas. Representatives of this genus are widespread in temperate North America, especially the southeastern and western United States, and in Siberia, the Himalayas, China, and Japan. They inhabit semidry areas, foothills, sandy washes, and stream banks. The plants are often found at altitudes up to 3,600 m. The drought-resistant feature of Thermopsis species, their deep-rooting habit, and their spreading by underground rootstocks to form broad patches are effective in erosion control. Thermopsis montana is an excellent soil-binder in the yellow pine belt of the western United States. Thermopsis caroliniana is commonly used as a bright perennial ornamental. Thermopsis lanceolata is used as an expectorant herb in the Ukraine [29].

Thermopsis rhombifoila, false lupine, a common herb of the dry plains of Canada southward and eastward to Nebraska and Colorado, has a common name of golden-bean or buffalo-bean [29]. It is an erect, branched perennial 15 - 50 cm high, usually in large patches, from running rootstocks. Leaves are compound with 3 obovate leaflets of about 2 - 4 cm long, covered with appressed silky gray hairs. Leaf stalks are large and have leaf-like stipules at the junction

of the stem. The flowers are very bright golden yellow of 1 - 2 cm long, in rather dense racemes; stamens separate. The pods are curved, grayish and hairy of 3 - 7 cm long, and contain 10 - 13 seeds. It is an early blooming plant and gives one of the most striking and colorful early spring flowers. It can be commonly found in great masses along roadsides, on edges of buffalo wallows, and on hillsides throughout the North America prairies, and also in sandy areas in parklands [30].

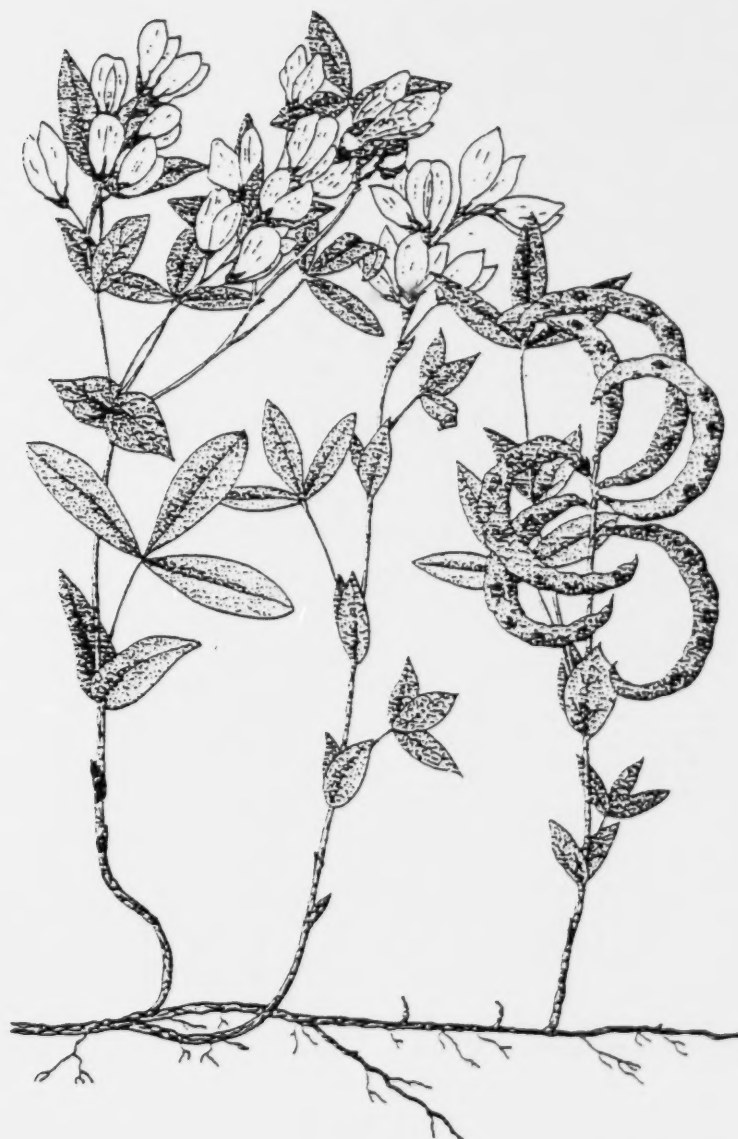


Figure 4. Buffalo-bean / Golden-bean, Thermopsis rhombifolia [29].



Figure 5. Distribution of Thermopsis rhombifolia in North America [30].

Thermopsis rhombifolia contains quinolizidine alkaloids [3]. Cattle have the capability to smell and/or taste the plant; thus, if given a choice, they will avoid the high-alkaloid-containing plants and consume other plants. Consequently, Thermopsis rhombifolia lacks forage qualities and gives us an advantage in locating and/or harvesting the plant for phytochemical investigation.

E. Alkaloidal constituents in Thermopsis species

Thermopsis is a Greek word, in which thermos means "a lupin" and -opsis indicates a "resemblance" [28]. Thus, species named under Thermopsis genus contain lupin-like characteristics.

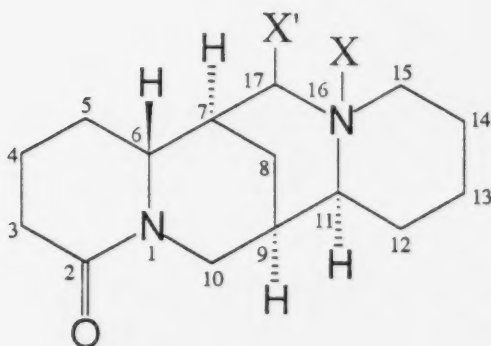
Manske and Marion [3] obtained three known alkaloids, thermopsine, N-methylcytisine, and cytisine from Thermopsis rhombifolia in 1943. Two other alkaloids, which they named rhombifoline ($C_{15}H_{20}O_2N_2$) and rhombinine ($C_{16}H_{22}O_2N_2$), were characterized as their perchlorates and picrates. The free bases of the latter two alkaloids have not been obtained in crystalline form and the assigned empirical formulae are regarded as tentative.

In 1969, Keller and Cole [32] studied the alkaloids of Thermopsis montana collected in Idaho. Cytisine, hydroxylupanine, N-methylcytisine, anagyrine and thermopsine were isolated and identified. Trace quantities of lupanine and 17-oxosparteine were tentatively identified, while sparteine and genisteine were absent.

Cytisine has been isolated from Thermopsis chinensis (Thermopsis fabaceae) [33]. Further chemical examination of the alkaloids of this species collected in the Okinoerabnjima region of Japan by Ohmiya and Otomasn in 1974 has resulted in the isolation of a total of five alkaloids: N-methylcytisine (0.042 %), cytisine (0.005 %), anagyrine

(0.025 %), lupanine (trace), and N-formylcytisine (0.0007 %) [33].

Thermopsis lupinoides has been used as a medicinal plant (ye jue ming) in China [34] and was considered a rich source of lupine alkaloids. Its seeds contain (+)-lupanine N-oxide (Figure 6(I)), together with nine other known lupine alkaloids, namely (+)-lupanine (Figure 6 (II)), (+)-17-oxolupanine (Figure 6(III)), (-)-anagyrine (Figure 2 (VIIIa)), (-)-baptifoline (Figure 2(VIIIb)), (-)-cytisine (Figure 2(IXa)), (-)-N-methylcytisine (Figure 2(IXb)), (-)-N-formylcytisine (Figure 6(IV)) and ammodendrine which were characterized by K. Saito et al. [35] in 1988.

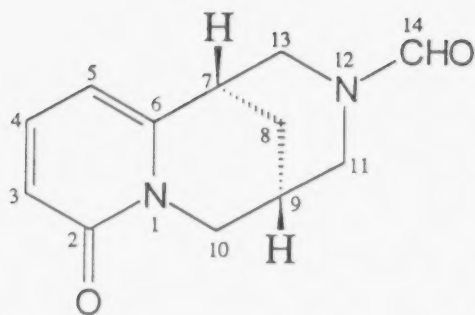


(I) (+)-lupanine N-oxide : $X=O$, $X'=H_2$

(II) (+)-lupanine : $X=\text{lone pair}$, $X'=H_2$

(III) (+)-17-oxolupanine : $X=\text{lone pair}$, $X'=O$

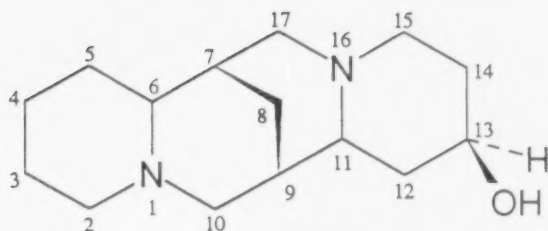
Figure 6. Alkaloids of Thermopsis lupinoides.



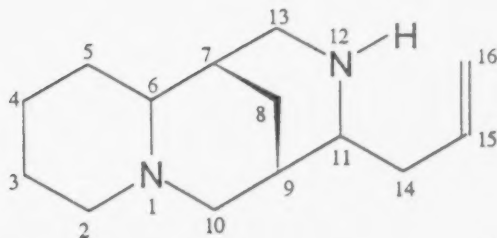
(IV) (-)-N-formylcytisine

Figure 6. (continued)

Christov et al. [36] discovered two novel quinolizidine alkaloids, 13-*epi*-hydroxysparteine (Figure 7(I)) and desoxyangustifoline (Figure 7(II)), from the aerial parts of the uninvestigated species Thermopsis mongolica collected in Baishint, Mongolia. (+)-Sparteine, 17-oxosparteine, 5,6-dehydrolupanine, α -isolupanine, (+)-hydroxysparteine, (-)-anagyrine, (-)-thermopsine, (-)-cytisine, and (-)-N-methylcytisine have been found in this plant.



(I) 13-*epi*-hydroxysparteine



(II) desoxyangustifoline

Figure 7. Two novel alkaloids of Thermopsis mongolica.

In addition, alkaloids like argentine, dimethamine, N-acetylcytisine, thermopsidine and tinctorine can be found in Thermopsis alterniflora; alkaloids like 13-hydroxylupanine can be found in Thermopsis cinerea, and alkaloids like dithermamine, homothermopsine, rhombifoline, thermopsamine, (-)-thermopsine can be found in Thermopsis lanceolata [37].

F. Toxicity and potential use of the lupine alkaloids in
Thermopsis rhombifolia

The toxicity of Thermopsis rhombifolia to humans and domestic animals has been recognized for some time. Accidental ingestion of the seeds was reported to cause poisoning in children. Thermopsis rhombifolia is toxic to cattle and may cause teratogenic deformities in calves [38].

Quinolizidine alkaloids with 2-pyridone A-ring, e.g. cytisine, may be responsible for the lesions and individual 2-pyridones may have additive effects. Grazing by pregnant cows has been shown to lead to congenital deformities known as "crooked calf disease" [39].

The major alkaloids, cytisine and N-methylcytisine, found in Mescal Bean produced abnormal behaviour in animals which closely resembles patterns produced by known hallucinogenic drugs, e.g. mescaline, N,N-dimethyltryptamine and psilocybin [40].

Cytisine and N-methylcytisine are lupine alkaloids found in Thermopsis rhombifolia in which cytisine falls in the same pharmacological group as nicotine. Cytisine has been described as "qualitatively indistinguishable" from nicotine. Cytisine is a powerful poison causing nausea, then convulsions, and death due to respiratory failure. It also causes microscopic skeletal muscle degeneration and necrosis. Cytisine is a potent nicotinic agonist in vitro,

a substrate and inhibitor of acetylcholine esterase. It has greater activity in stimulating ganglia than in blocking ganglia [41].

The toxicity of N-methylcytisine was examined [42] and its pharmacological properties were briefly reported [43]. Although its peripheral actions resembled those of nicotine, the convulsions produced by N-methylcytisine in mice differed from those produced by nicotine. Studies on mice done by Barlow and McLeod [44] indicated that convulsions produced by the ingestion of cytisine and N-methylcytisine were much slower in onset and much less severe than those of nicotine. In most tests, cytisine is active in doses from a quarter to three-quarters of those of nicotine; N-methylcytisine is active in doses from 10 to 20 times those of cytisine. Cytisine and N-methylcytisine may differ from nicotine in their central effects and they are active as the cation forms. The pK_a of cytisine is 7.92 and that of N-methylcytisine is 7.04; the difference accounts, in part, for the weaker activity of N-methylcytisine. The N-methylcytisine ion is generally one-sixth to one-third as active as the cytisine ion.

Table 2. Comparison of the pharmacological action of N-methylcytisine and nicotine [43].

Test	Result
Action on frog heart ganglia	Nicotine is 25X as potent as N-methylcytisine
Circulatory action in dogs	Nicotine is 10X as active as N-methylcytisine
Respiratory stimulation through carotid sinus	Nicotine is 10X as active as N-methylcytisine
Effect on rabbit's isolated small intestine	Nicotine's activity is 10X as great as N-methylcytisine
Hyperglycemic action in rabbits	Nicotine's dose of 1 mg/kg = 20 mg/kg of N-methylcytisine
Toxicity (the median lethal dose) in mice	Nicotine is 40X as toxic as N-methylcytisine

Cytisine is a ganglionic agonist which competes with high affinity for brain nicotinic cholinergic receptors. Its binding is high in the thalamus, striatum, and cortex than in the hippocampus, cerebellum, or hypothalamus. The high affinity and low nonspecific binding nature of cytisine make it a useful ligand for studying neuronal nicotinic receptors [44].

PART THREE

EXPERIMENTAL

A. Instruments

1. Automatic fraction collector

(Buchler Fractomette 220, Fort Lee, New Jersey)

2. Circular polarimeter (0.01°)

(CARL ZEISS, Oberkochen/Wuertt., Germany)

3. Continuous extractor

(Corning, New York)

4. Digital pH meter

(Corning pH meter 220, New York)

5. Electronic analytical balance

(Sartorius Instrument Ltd., Surrey, Great Britain)

6. Flash evaporator

(Buchler Instruments, Fort Lee, New Jersey)

7. Glass column

(Kontes Glass Co., Vineland, New Jersey)

8. Heavy duty blender

(Waring Products Division, New Hartford, Connecticut)

9. High performance liquid chromatography analytical column
(Millipore, Waters Nova-Pak silica C₁₈ steel column, 4
μm, 3.9 mm x 150 mm, Milford, Massachusetts)
10. High performance liquid chromatography injector
(Millipore, Waters Model U6K, Milford, Massachusetts)
11. High performance liquid chromatography integrator
(Hewlett Packard 3390A, Hewlett Packard Co., Avondale,
Pennsylvania)
12. High performance liquid chromatography system
(Waters Associates, Milford, Massachusetts)
 - a) Solvent delivery system (Waters model 501)
 - b) Detector (Waters model 468)
13. Hot-stage melting point apparatus
(Mettler Instrument Corporation, Princeton, New Jersey)
14. Infrared spectrophotometer
(Nicolet FT, Nicolet Instrument Corporation, Madison,
Wisconsin)
15. Micro Hammer-Cutter mill
(Glen mills, Clifton, New Jersey)

16. Nuclear magnetic resonance spectrophotometer
(Bruker AM300 FT, Spectrospin, Toronto, Canada)
17. Reagent sprayer
(CAMAG, Schott, Berlin, West Germany)
18. Thin-layer chromatogram chamber
(Eastman Kodak Co., Rochester, New York)
19. Thin-layer chromatogram glass developing tank
(CAMAG, Schott, Berlin, West Germany)
20. Triple beam balance
(Ohaus Scale Corp. Union, Florham Park, New Jersey)
21. Ultra-violet lamp
(Mineralight UV lamp model UVGL-58, Ultra-violet
Products Inc., San Gabriel, California)
22. Ultra-violet/Visible spectrophotometer
(Philips PU8740, Pye Unicam Ltd., Cambridge, Great
Britain)

B. Materials

1. Acetone

(Anachemia Chemicals Inc., Rouses Point, New York)

2. Acetonitrile, HPLC grade, UV cutoff 190nm

(Fisher Scientific, Ottawa, Ontario)

3. Aerial parts of Thermopsis rhombifolia

- TrI : collected at Tofield of Alberta on July 14, 1991
- TrII : collected at Killam of Alberta on May 29, 1993
- TrIII : collected at Ponoka of Alberta on July 13, 1994

4. Ammonia solution, 29.5 % (NH₃)

(BDH, Dorset, England)

5. (-)-Cytisine

(Sigma Chemical Co., St. Louis, Missouri)

6. Deuteriochloroform, 99.8 atom % D

(Sigma Chemical Co., St. Louis, Missouri)

7. Diatomaceous earth

(Celite® 545, Fisher Scientific, Ottawa, Ontario)

8. Dichloromethane

(Fisher Scientific)

9. Ethanol, 95%
(Commercial Alcohols Inc., Montreal-Toronto)
10. Ethanol, 98%
(Commercial Alcohols Inc., Montreal-Toronto)
11. Ethyl acetate
(Anachemia, Chemicals Inc., Rouses Point, New York)
12. Filter paper
(Whatman, Clifton, New Jersey)
13. Glacial acetic acid
(BDH, Dorset, England)
14. Hydrochloric acid, 36.5 - 38.0 %
(BDH, Dorset, England)
15. Membrane filter ,Nylon-66, 0.45 μ m pore size, 47 mm
diameter
(PALL, East Hills, New York)
16. Methanol (MeOH), anhydrous
(Mallinckrodt, Chesterfield, Missouri)

17. Potassium bromide, IR grade
(BDH, Dorset, England)
18. Potassium phosphate, monobasic
(Baker Chemical Co., Sanford, Maine)
19. Pre-coated analytical silica gel TLC plate with
fluorescent indicator, 0.1 mm thickness
(Eastman Kodak Co., Rochester, New York)
20. Silicic acid 100 mesh
(Mallinckrodt, Chesterfield, Missouri)
21. Sodium phosphate, dibasic anhydrous
(American Scientific and Chemical, Natick,
Massachusetts)
22. Washed sea sand
(Fisher Scientific, Ottawa, Ontario)

* All solvents meet A.C.S. specifications

C. Reagents

1. Dragendorff's reagent [45]

A stock solution was made by boiling 2.6 g of bismuth subcarbonate and 7.0 g of dry sodium iodide in 25 ml of glacial acetic acid for a few minutes, allowing the solution to cool overnight, and filtering and mixing 20 ml of the clear filtrate with 80 ml of ethyl acetate. The spraying reagent was made by mixing 10 ml of the above stock solution with 50 ml of glacial acetic acid and 120 ml of ethyl acetate, and then stirring with 10 ml of distilled water.

(Alkaloids are detected on chromatograms with Dragendorff's spray reagent as orange to red spots on a light orange background. The background may be decolorized by spraying with a 2 % aqueous acetic acid solution.)

2. Ethanol, 75 % (V/V)

It was prepared by mixing 95 % ethanol and distilled water in a ratio of 75 : 20 (V/V).

3. Hydrochloric acid solution, ca. 1 M

49.3 ml of concentrated hydrochloric acid (36.5 - 38.0 %) was diluted to 500.0 ml with distilled water in a volumetric flask.

4. Iodoplatinate acid reagent [46]

Three ml of a 10 % solution of chloroplatinic acid was mixed with 97 ml of distilled water and 100 ml of 6 % potassium iodide was added.

(Alkaloids are detected on chromatograms with iodoplatinate acid spray reagent as grey to violet or blue spots on a pink to purple background.)

5. Mayer's reagent [47]

1.36 g of mercuric chloride and 5.00 g of potassium iodide were dissolved in 100 ml distilled water.

(Alkaloids are detected in solution with Mayer's reagent as a cream-colored precipitate.)

6. Phosphate buffer solution (pH = 5.52) [48]

9.073 g of monobasic potassium phosphate was dissolved in distilled water and diluted to exactly 1000 ml in a volumetric flask (stock A). 9.464 g of anhydrous dibasic sodium phosphate was dissolved in distilled water and diluted to exactly 1000 ml in a volumetric flask (stock B). The final buffer solution was prepared by mixing stock A with stock B in a ratio of 100 : 6 (V/V).

D. Extraction of the basic constituents from Thermopsis
rhombifolia

D.1. Sample pretreatment

The three samples, TrI, TrII and TrIII were individually studied as described below.

D.1.a. For sample TrI

The aerial parts (with seeds) were air-dried first, chopped up into small pieces and then ground into powdered form with a micro hammer-cutter mill in a fumehood.

D.1.b. For sample TrII

The fresh aerial parts (with flowers) were chopped up into small pieces and preserved with 75 % ethanol (V/V) at 4°C.

D.1.c. For sample TrIII

The fresh aerial parts (with seeds) were chopped up into small pieces and preserved with 75 % ethanol (V/V) at 4°C.

D.2. Sample extraction

The pretreated samples TrI (300 g), TrII (1000 g) and TrIII (1000 g) were soaked in 75 % ethanol (V/V) and extracted by mixing in a blender. The extract was drained off and the marc, after drying, was again extracted by adding fresh 75 % ethanol (V/V). The extraction process was repeated until no signs of alkaloids were detected by adding a few drops of Mayer's reagent to a small portion of the extract lastly obtained (ca. 8 L of 75 % ethanol (V/V) was used in total in each of the samples).

The combined ethanolic extract for each sample was filtered by suction pump and was concentrated under reduced pressure at 40°C by the flash evaporator to a thick dark syrup. The concentrated extract was acidified by adding ca. 1 M hydrochloric acid until pH = 1 (measured by digital pH meter). The acidified extract was stirred until the gummy substance dissolved. It was then filtered by suction pump and the brown residue was redissolved in 75 % ethanol and extracted until there were no signs of alkaloids present when Mayer's reagent was used.

The clear filtrate was transferred into a continuous extractor and extracted with dichloromethane for 24 - 48 hours. The aqueous basic fraction was collected and basified by adding cold ammonia solution (29.5 %, W/V) until pH = 8 (measured by digital pH meter) and then continuously

extracted with dichloromethane. The dichloromethane extract was evaporated to dryness under reduced pressure and weighed.

A summary of the extraction procedure is presented in Figure 8.

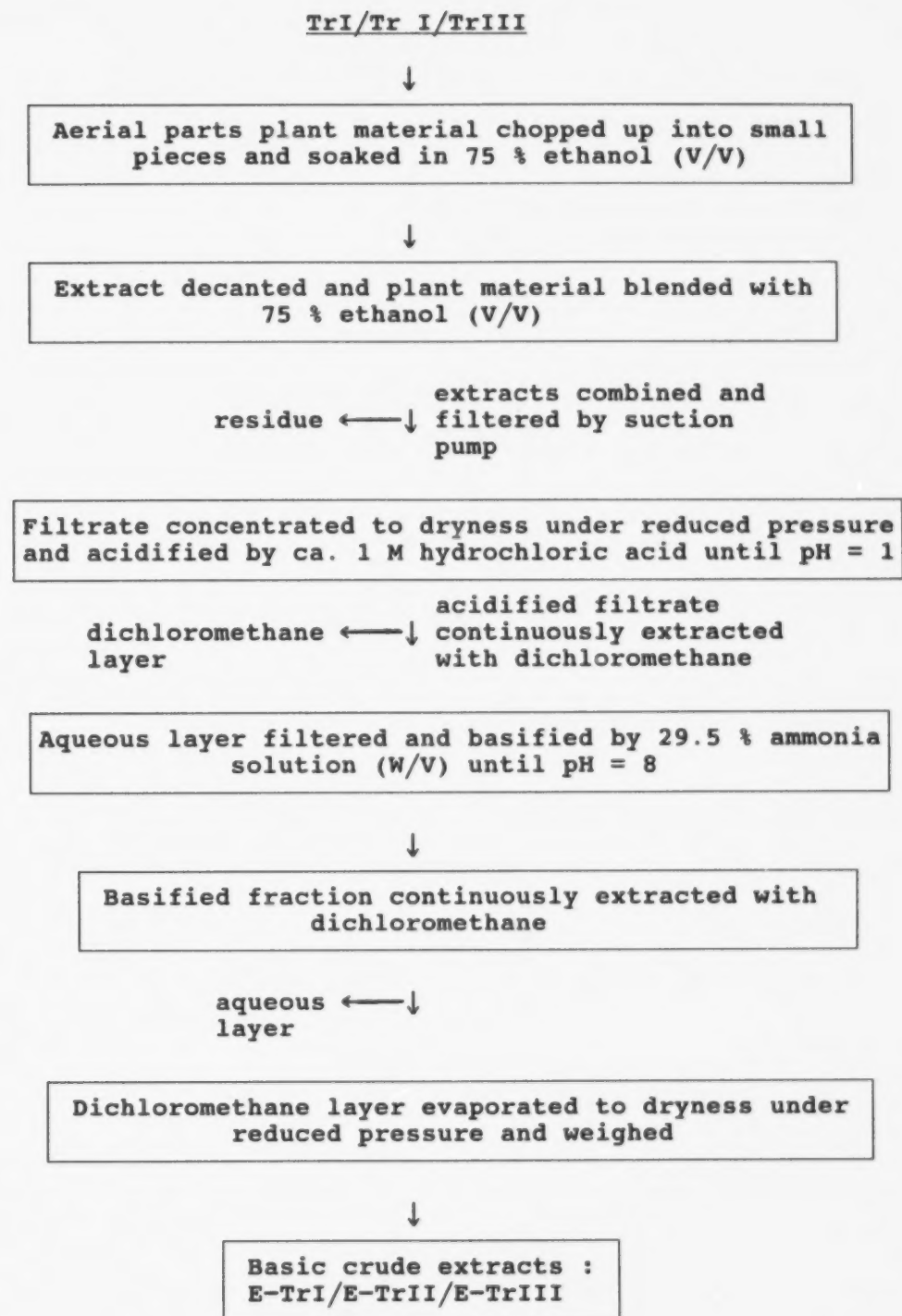


Figure 8. Extraction scheme for Thermopsis rhombifolia.

E. Isolation of the alkaloidal constituents from Thermopsis rhombifolia

Portions of the crude extract E-TrI were separated by column chromatography with the following specifications :

Bulb volume of the column	= 500 ml
Total volume of the column	= 625 ml
Internal diameter of the column	= 1.98 cm
Weight of adsorbent material used	= 60.0 g
Composition of adsorbent material	= 80 : 30
(Silicic acid : Celite®)	
Length of adsorbent matrix	= 38 cm

The chromatographic column was gravity packed (with occasional tapping) by filling with the slurry made from mixing 60 g adsorbent material with 100 ml of the solvent system : CH_2Cl_2 /acetone/MeOH/29.5 % NH_3 (53 : 40 : 5 : 0.9).

Sufficient glass wool was plugged at the column outlet and 50 ml of the solvent system was added to the column prior to its packing. Excess solvent was allowed to drain through the column outlet, leaving enough solvent to just cover the gel surface.

The sample was dissolved in a minimal amount of the solvent system and applied onto the surface of the gel bed by a long dropper. After the sample had completely migrated

into the adsorbent material, more solvent was added on top of the gel surface which was then covered with ca. 0.3 cm of washed sea sand. The column was run by gradient elution with a mixture of solvents (CH_2Cl_2 /acetone/MeOH/ammonia solution) in order of increasing polarity as shown in Table 3.

Table 3. Solvent compositions for the gradient elution column chromatography of the basic crude extract from Thermopsis rhombifolia.

Solvent system	* Dichloro-methane	* Acetone	* Methanol	* Ammonia 29.5 %	Total volume (ml)
S1	53	40	5	0.9	300
S2	50	40	8	0.9	200
S3	46	40	12	0.9	200
S4	40	40	18	0.9	200

* the numbers are expressed as the volume ratio of individual solvent in the solvent system used.

The flow rate was manually adjusted to one ml per minute, and fractions of 10 ml each were collected by an automatic fraction collector. The fractions collected were monitored on the basis of thin-layer chromatography (TLC) patterns using ultraviolet light and iodoplatinate reagent/ Dragendorff's reagent.

Fractions which gave spots of the same R_f value(s) and colour characteristics in the TLC analysis were combined for further analysis. The fractions combined and the corresponding R_f values were summarized in Table 6. The combined fractions were further purified by repeated column chromatography.

Table 4. Data for the isolation of E-TrI by silica column chromatography.

Fraction no.	TLC (Rf values)	UV (short wave)	Colour (Dragendorff's)
1 - 5	-	-	-
6 - 15	0.82 (#1), 0.77 (#2)	quenching	orange
16 - 27	0.77 (#2), 0.73 (#3)	quenching	orange
28 - 34	0.73 (#3)	quenching	orange
35 - 37	-	-	-
38 - 43	0.52 (#4)	quenching	orange
44 - 46	-	-	-
47 - 66	0.40 (#5)	quenching	orange

Note:

- 1) Chromatograms were developed with precoated silica gel plates and the solvent system :
CH₂Cl₂/acetone/MeOH/29.5 % Ammonia (44:40:14:0.9)
- 2) Quenching indicates that the compound absorbs ultraviolet radiation and gives dark appearance on the TLC chromatogram.

E.1. The less polar group included; fractions 6 - 15 (contained alkaloids #1 and #2), fractions 16 - 27 (contained alkaloids #2 and #3), and fractions 28 - 34 (contained alkaloids #3).

Each fraction was concentrated to dryness under reduced pressure and rechromatographed on a column with the following specifications:

Bulb volume of the column	= 200 ml
Total volume of the column	= 222 ml
Internal diameter of the column	= 1.07 cm
Weight of adsorbent material used	= 10.5 g
Composition of adsorbent material (Silicic acid : Celite®)	= (80 : 30)
Length of adsorbent matrix	= 23 cm

The chromatographic column was gravity packed (with occasional tapping) by filling with the slurry made from mixing 10.5 g adsorbent material with 50 ml of the solvent system : CH_2Cl_2 /acetone/MeOH/29.5 % NH_3 (60 : 35.5 : 2.5 : 0.9). The column was run by isocratic elution with the same solvent system.

Complete separation of alkaloid #1 from alkaloid #2 was successful after two more columns were run. Complete

separation of alkaloid #2 from #3 was successful after two more columns were run. Complete separation of alkaloid #3 from the colouring matter was successful after one more column was run.

E.2. The more polar group included fractions 38 - 43 (contained alkaloid #4), fractions 47 - 66 (contained alkaloid #5), and fractions 28 - 34 (contained alkaloid #3)

Each fraction was concentrated to dryness under reduced pressure and rechromatographed on a column with the following specifications:

Bulb volume of the column	= 250 ml
Total volume of the column	= 350 ml
Internal diameter of the column	= 1.91 cm
Weight of adsorbent material used	= 38.0 g
Composition of adsorbent material (Silicic acid : Celite®)	= (80 : 30)
Length of adsorbent matrix	= 30 cm

The chromatographic column was gravity packed (with occasional tapping) by filling with the slurry made from mixing 38.0 g adsorbent material with 100 ml of the solvent system : CH_2Cl_2 /acetone/MeOH/29.5 % NH_3 (44 : 40 : 14 : 0.9).

The column was run by isocratic elution with the same solvent system.

Complete separation of alkaloid #4 and alkaloid #5 was successful after one more column was run for each of them.

E.3.

After extensive purification of the alkaloidal constituents, the corresponding alkaloid components were combined and concentrated to dryness under reduced pressure. Each of them was applied individually on precoated TLC plates and confirmed to be a single spot for each of the five isolated components. In addition, co-TLC of alkaloid #5 with the authentic sample of cytosine was done. Refer to Figure 9 for a summary of the TLC data.

E.3.a. Alkaloid #1 (TLC R_f value = 0.82)

Solvent was removed in vacuum under reduced pressure at 40°C and the residue was treated with cold acetone. Crystals were grown by slow evaporation from acetone. After several recrystallizations and drying in a dessicator, alkaloid #1 was obtained as light yellow crystals and the melting point was measured by a hot-stage melting point apparatus.

E.3.b. Alkaloid #2 (TLC R_f value = 0.77)

Several different solvents and mixtures of solvents were tried to induce the growth of crystals, but failed (possibly due to sample degradation or the small amount of alkaloid #2 that was available from the plant material). Alkaloid #2 was obtained in the form of a yellowish oil.

E.3.c. Alkaloid #3 (TLC R_f value = 0.73)

Solvent was removed in vacuum under reduced pressure at 40°C and the residue was treated with a minimal amount of benzene. By several repetitions of this procedure and drying in a desiccator, alkaloid #3 was obtained as colourless needles and the melting point was measured by a hot-stage melting point apparatus.

E.3.d. Alkaloid #4 (TLC R_f value = 0.52)

Several different solvents and mixtures of solvents have been used to induce the growth of crystals, but failed (possibly due to sample degradation or the small amount of alkaloid #4 available in the plant material). Alkaloid #4 was also obtained in the form of a yellowish oil.

E.3.e. Alkaloid #5 (TLC R_f value = 0.40)

Solvent was removed in vacuum under reduced pressure at 40°C and the residue was treated with cold acetone. When the solution was allowed to stand for hours, colourless crystals

were formed. After repeated recrystallization from acetone, the melting point of alkaloid #5 and the mixed melting point of it with the authentic sample of cytosine were measured by a hot-stage melting point apparatus. Refer to Table 7 for a summary of the melting point data.

F. Optical rotations of the isolated alkaloids from
Thermopsis rhombifolia

The samples were prepared by dissolving a desired accurately weighed amount of the isolated alkaloids in 98% ethanol or distilled water. The samples were made up to mark in volumetric flasks to bring the alkaloid concentrations to the experimental range for optical rotation measurements.

The observed angle of optical rotation of each alkaloid was determined by using the sodium D line (wavelength = 589.3nm) with a cell length of 0.5 dm at 25°C.

G. Spectrophotometric methods

G.1. Infrared spectroscopy (IR)

G.1.a. KBr disc

Alkaloids #1, #3 and #5 were prepared in the form of KBr pellets by mixing ca. 1 mg crystallized sample with ca. 40 mg IR grade KBr solid in a mill and then compressed under vacuum and high pressure.

G.1.b. Neat

Alkaloids #2 and #4 were prepared by applying two drops of the sample onto a KBr disc and then sandwiched by another matched KBr disc. Each sample was scanned by the Infrared Spectrophotometer in the range from 600 cm^{-1} to 3800 cm^{-1} with air as the blank. Refer to Tables 8-12 for a summary of the IR data.

G.2. Nuclear Magnetic Resonance spectroscopy (NMR)

G.2.a. ^1H NMR spectroscopy

^1H NMR (300 MHz) spectra were recorded for each isolated alkaloid by using deuteriochloroform as the solvent. Refer to Tables 13,15,17,19 and 21 for a summary of the ^1H NMR data for each alkaloid.

G.2.b. ^{13}C NMR spectroscopy

^{13}C NMR (75 MHz) spectra were recorded for each isolated alkaloid by using deuteriochloroform as the solvent. Refer to Tables 14,16,18,20 and 22 for a summary of the ^{13}C NMR data for each alkaloid.

G.3. Ultraviolet spectroscopy (UV)

The samples were prepared by dissolving a desired accurate amount of the isolated alkaloids in distilled water. Serial dilutions were made in volumetric flasks to bring the alkaloid to the optimum concentrations for UV measurements. The UV spectrum for each alkaloid was scanned in the range from 200 nm to 350 nm with distilled water as the blank. Refer to Table 23 for a summary of the UV data for each alkaloid.

H. High Performance Liquid Chromatography (HPLC)

H.1. Standards preparation

The purified alkaloids were obtained from sample TrI and the corresponding standard solutions were prepared as shown below for use in quantitative analysis by HPLC.

H.1.a. Standard alkaloid #1

The injection samples were prepared by dissolving 5.0 mg of the purified alkaloid #1 in distilled water and diluted to mark in a 50.0 ml volumetric flask. From this concentration of 100 ppm, serial dilutions of 30 ppm, 20 ppm, 15 ppm, 10 ppm, and 5 ppm were made.

H.1.b. Standard alkaloid #3

The injection samples were prepared by dissolving 5.0 mg of the purified alkaloid #3 in distilled water and diluted to mark in a 50.0 ml volumetric flask. From this concentration of 100 ppm, serial dilutions of 20 ppm, 15 ppm, 10 ppm, 8 ppm, and 5 ppm were made.

H.1.c. Standard alkaloid #5

The injection samples were prepared by dissolving 5.0 mg of the purified alkaloid #5 in distilled water and diluted to mark in a 50.0 ml volumetric flask. From this concentration of 100 ppm, serial dilutions of 15 ppm, 10 ppm, 8 ppm, 5 ppm, and 3 ppm were made.

H.2. Sample preparation

Each sample was first fractionated into less polar and more polar groups in order to remove undesired impurities that might be present in the crude extract and to allow more flexibility when determining for the optimum HPLC conditions to give the best resolution of the peaks.

H.2.a. Sample TrI

The basic crude extract (TrI, 24.8 mg) was fractionated into two groups by column chromatography {stationary phase, silicic acid : Celite® = 80 : 30; mobile phase : CH_2Cl_2 /acetone/MeOH/29.5% NH_3 (44 : 40 : 14 : 0.9)}.

The less polar group (alkaloids #1, #2 and #3) was dissolved in distilled water and diluted to mark in a 25.0 ml volumetric flask. The injection samples were prepared by pipetting 1.0 ml of the above solution into 10.0 ml and 20.0 ml volumetric flasks containing distilled water to give 10-fold and 20-fold dilution solutions respectively.

The more polar group (alkaloids #4 and #5) was prepared in a similar way as the less polar group.

H.2.b. Sample TrII

The basic crude extract (TrII, 231.5 mg) was fractionated into two groups by column chromatography (stationary phase, silicic acid : Celite® = 80 : 30; mobile phase : CH_2Cl_2 /acetone/MeOH/29.5% NH_3 (44 : 40 : 14 : 0.9)).

The less polar group (alkaloids #1, #2 and #3) was dissolved in distilled water and diluted to mark in a 250.0 ml volumetric flask. The injection samples were prepared by pipetting 1.0 ml of the above solution into 5.0 ml and 10.0 ml volumetric flasks containing distilled water to give 5-fold and 10-fold dilution solutions respectively.

The injection samples for the more polar group (alkaloids #4 and #5) were prepared in a similar manner but giving 10-fold and 40-fold dilution solutions.

H.2.c. Sample TrIII

The basic crude extract (TrIII, 335.0 mg) was fractionated into two groups by column chromatography ((stationary phase, silicic acid : Celite® = 80 : 30; mobile phase : CH_2Cl_2 /acetone/MeOH/29.5% NH_3 (44 : 40 : 14 : 0.9)).

The less polar group (alkaloids #1, #2 and #3) was dissolved in distilled water and diluted to mark in a 100.0 ml volumetric flask. The injection samples were prepared by serial dilutions to give a final solution which is 75-fold more dilute than the above solution. The more polar group (alkaloids #4 and #5) was dissolved in distilled water and diluted to mark in a 100.0 ml volumetric flask. The injection sample was prepared by serial dilutions to give a final solution which is 20-fold more dilute than the above solution.

H.3. Quantitative analysis of the alkaloidal constituents

High performance liquid chromatography was carried out with the HPLC system described in the instrumentation section. The mobile phase was filtered through a 0.45 μm membrane filter before use and was sparged with helium gas during analysis. The injection volumes were all 10 μl and the HPLC conditions for the separation of and quantification of the lupine alkaloids are summarized in Table 5.

Table 5. HPLC conditions for the analysis of the lupine alkaloids in Thermopsis rhombifolia.

Mode	Column	*Solvent	Flow rate (ml/min)	Detection (nm)	Application (alkaloids)
Reversed phase	Silica C ₁₈ , 150x3.9mm I.D., 4 μ m particle size	a	1.2	310	#1, #2 and #3
Reversed phase	Silica C ₁₈ , 150x3.9mm I.D., 4 μ m particle size	b	1.0	310	#4 and #5

* a = CH₃CN/phosphate buffer, pH = 5.52 (13:1)

b = CH₃CN/phosphate buffer, pH = 5.52 (35:1)

See Figures 14 and 15 for the HPLC profiles of the alkaloid constituents in Thermopsis rhombifolia.

Calibration curves for alkaloids #1, #3 and #5 were generated by plotting the peak areas versus concentrations (ppm) calculated from linear regression. Refer to Tables 24-26 and Figures 16-18 for a summary of the calibration curve data.

The amount of each alkaloid in the plant crude extract was determined by an external standard method or an area normalization method.

PART FOUR

RESULTS AND DISCUSSION

A. Extraction of the basic constituents

After extensive solvent extraction of the basic constituents from the sample, the crude extract was evaporated to dryness under reduced pressure at 40°C and the residue obtained was weighed accurately. The results are presented in Table 6.

Table 6. Comparison of the yields of the basic crude extracts of Thermopsis rhombifolia.

Sample	% W/W yield of basic crude extract
TrI	0.58 (dry weight)
TrII	0.23 (fresh weight)
TrIII	0.34 (fresh weight)

By comparing the % yield of the basic crude extract, sample TrIII has a slightly higher yield than that of sample TrII. However, the exceptionally high yield of the basic crude extract in sample TrI is due to the removal of water in the sample pretreatment process of TrI.

B. Thin-layer chromatography analysis

solvent front

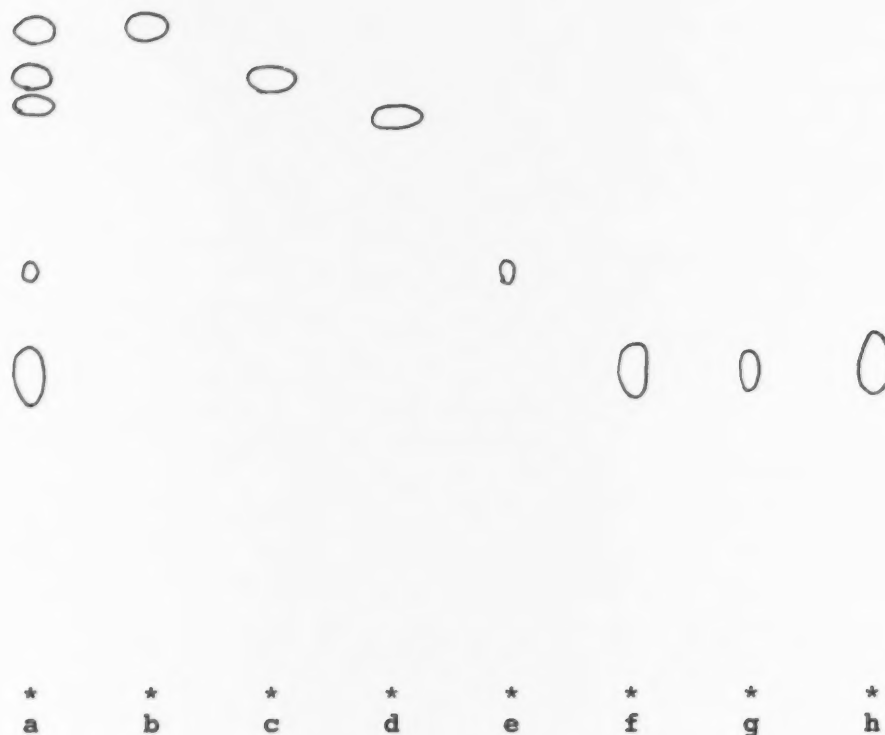


Figure 9. Thin-layer chromatogram for the alkaloids of Thermopsis rhombifolia from E-TrI.

* : point of application a : crude extract E-TrI
b : alkaloid #1 c : alkaloid #2
d : alkaloid #3 e : alkaloid #4
f : alkaloid #5 g : cytisine
h : co-TLC of alkaloid #5 and cytisine

(solid lines indicate strong positive reaction for alkaloids after spraying with Dragendorff's reagent)

Chromatograms were developed with the solvent system :
 $\text{CH}_2\text{Cl}_2/\text{acetone}/\text{MeOH}/29.5\% \text{ NH}_3$ (44:40:14:0.9)

Five distinct alkaloids were individually isolated and alkaloid #5 was found to have an R_f value to that of authentic sample of (-)-cytisine.

Table 7. TLC R_f data for the isolated alkaloids of Thermopsis rhombifolia from E-TrI.

Sample	TLC R_f value
Alkaloid #1	0.82
Alkaloid #2	0.77
Alkaloid #3	0.73
Alkaloid #4	0.52
Alkaloid #5	0.40
Cytisine	0.40
#5 + Cytisine	0.40

From the TLC R_f values, the degree of retention of the isolated alkaloids by the stationary phase is in the increasing order of alkaloid #1, alkaloid #2, alkaloid #3, alkaloid #4 and alkaloid #5. Since the samples were developed under normal phase conditions, the polarity of the isolated alkaloids should be in the increasing order of alkaloid #1, alkaloid #2, alkaloid #3, alkaloid #4 and alkaloid #5.

C. Melting point analysis

Table 8. Melting point data for the isolated alkaloids of Thermopsis rhombifolia from E-Tri.

Sample	Melting point (°C, uncorrected)
Alkaloid #1	205.0 - 205.2
Alkaloid #2	oil
Alkaloid #3	136.0 - 137.6
Alkaloid #4	oil
Alkaloid #5	153.5 - 154.0
Cytisine	153.0 - 155.0
#5 + Cytisine	153.0 - 154.5

The alkaloids melted sharply at the temperatures measured and alkaloid #5 was found to melt sharply at the same temperature either alone or in admixture with the authentic sample of (-)-cytisine.

Assignments:

The melting point of alkaloid #1 matches with that of thermopsine (205.5 - 206.5 °C) [49].

The melting point of alkaloid #3 matches with that of N-methylcytisine (136.0 - 138.0 °C) [50].

The melting point of alkaloid #5 matches with that of cytisine (153.0 - 155.0 °C) [51].

D. Optical activity analysis

Table 9. Optical activity data for the isolated alkaloids of Thermopsis rhombifolia from E-TRI.

Sample	Conc. (g/ml)	α (degree)	$[\alpha]_D^{25***}$ (degree)
Alkaloid #1	0.0478*	-3.7	-154.8
Alkaloid #2	#	levorotatory	levorotatory
Alkaloid #3	0.0854**	-9.4	-220.7
Alkaloid #4	#	levorotatory	levorotatory
Alkaloid #5	0.0450**	-2.7	-120.0
cytisine	0.0434**	-2.6	-119.8

Legend: * in ethanol

** in distilled water

*** $[\alpha]_D^{25} = \alpha / (l \times d)$

the exact concentration was not known

where d represents concentration in g/ml
l represents cell length in dm

The sign of optical rotation in each measurement was confirmed by diluting the sample to one-half of the stock concentration and repeating the measurement again.

Assignments:

The specific rotation of alkaloid #1 matches with that of (-)-thermopsine ($[\alpha]_D^{25} = -155$ degrees, ethanol) [49].

The specific rotation of alkaloid #3 matches with that of (-)-N-methylcytisine ($[\alpha]_D^{25} = -221$ degrees, water) [50].

The specific rotation of alkaloid #5 matches with that of (-)-cytisine ($[\alpha]_D^{25} = -119$ degrees, water) [51].

E. Infrared spectroscopy analysis

Table 10. IR absorption bands and assignments for alkaloid #1 of Thermopsis rhombifolia from E-TrI.

IR band (cm ⁻¹)	Assignment
2900-2950 (str, sh)	transquinolizidine, Bohlmann bands[52]
1646 (str, sh)	2-pyridone, C=O
1573 (str, sh)	2-pyridone, C-N
1548 (str, sh)	2-pyridone, C-N
1468 (wk, sh)	CH ₂ scissor
1100 (str, br)	aliphatic C-N stretch
749 (wk, sh)	olefinic C-H out-of-plane bend

Legend: str=strong, sh=sharp, wk=weak, br=broad

The characteristic IR peaks of alkaloid #1 match with the literature values for the lupine alkaloid, thermopsine [53].

See Appendix I for the original IR spectrum of alkaloid #1.

Table 11. IR absorption bands and assignments for alkaloid #2 of Thermopsis rhombifolia from E-TrI.

IR band (cm ⁻¹)	Assignment
2922 (str, sh)	transquinolizidine, Bohlmann bands[52]
2865 (med, sh)	C-H stretch
1650 (str, sh)	2-pyridone, C=O
1566 (str, sh)	2-pyridone, C-N
1549 (str, sh)	2-pyridone, C-N
1461 (wk, sh)	CH ₂ scissor
1359 (med, sh)	unknown
1207 (med, sh)	unknown
1143 (str, sh)	unknown
1068 (wk, sh)	unknown
798 (str, sh)	unknown

Legend: str=strong, sh=sharp, wk=weak, br=broad, med=medium

The characteristic IR peaks of alkaloid #2 show the presence of a 2-pyridone ring. See Appendix I for the original IR spectrum of alkaloid #2.

Table 12. IR absorption bands and assignments for alkaloid #3 of Thermopsis rhombifolia from E-TrI.

IR band (cm ⁻¹)	Assignment
2933 (str, sh)	transquinolizidine, Bohlmann bands[52]
2778 (str, sh)	C-H stretch
1650 (str, sh)	2-pyridone, C=O
1573 (str, sh)	2-pyridone, C-N
1551 (str, sh)	2-pyridone, C-N
1468 (med, sh)	CH ₂ scissor
1100 (str, br)	aliphatic C-N stretch
743 (wk, sh)	olefinic C-H out-of-plane bend

Legend: str=strong, sh=sharp, wk=weak, br=broad, med=medium

The characteristic IR peaks of alkaloid #3 match with the literature values for the lupine alkaloid, N-methylcytisine [53]. See Appendix I for the original IR spectrum of alkaloid #3.

Table 13. IR absorption bands and assignments for alkaloid #4 of Thermopsis rhombifolia from E-TrI.

IR band (cm ⁻¹)	Assignment
3100-3400 (str, br)	O-H, H-bonded
2800-3000 (str, br)	transquinolizidine, Bohlmann bands[52]
1647 (str, br)	2-pyridone, C=O
1547 (str, br)	2-pyridone, C-N
1371 (str, br)	unknown
1156 (str, br)	unknown
1035 (med, br)	unknown
962 (wk, sh)	unknown
805 (wk, sh)	unknown
734 (wk, br)	unknown

Legend: str=strong, sh=sharp, wk=weak, br=broad, med=medium

The characteristic IR peaks of alkaloid #4 show the presence of a 2-pyridone ring. See Appendix I for the original IR spectrum of alkaloid #4.

Table 14. IR absorption bands and assignments for alkaloid #5 of Thermopsis rhombifolia from E-TrI.

IR band (cm ⁻¹)	Assignment
3284 (str, sh)	N-H stretch
2943 (str, br)	transquinolizidine, Bohlmann bands [52]
1649 (str, sh)	2-pyridone, C=O
1565 (str, sh)	2-pyridone, C-N
1548 (str, sh)	2-pyridone, C-N
1480 (med, sh)	CH ₂ scissor
1104 (med, sh)	aliphatic C-N stretch
748 (str, br)	aromatic C-H out-of-plane bend

Legend: str=strong, sh=sharp, br=broad, med=medium

The characteristic IR peaks of alkaloid #5 match with the literature values for the lupine alkaloid, cytisine [53] and are superimposable on those of the authentic sample of (-)-cytisine. See Appendix I for the original IR spectrum of alkaloid #5.

F. Nuclear Magnetic Resonance Spectroscopy analysis

F.1. Alkaloid #1

All the ^1H NMR signals of alkaloid #1 were assigned as shown in Table 15 from analysis of a D_2O exchange experiment, a decoupling experiment [54] and comparison with the spectra data of cytosine-type alkaloids [35,53,55-58].

Table 15. ^1H NMR chemical shifts for alkaloid #1 in CDCl_3 .

Chemical shifts (δ , ppm)	No. of protons	Multiplicity (J, Hz)	Assignment (carbon no.) see Figure 10
7.25	1	dd, J=7.1 & 9.4	4
6.42	1	d, J=8.5	3
5.94	1	d, J=6.6	5
4.24	1	d, J=15.8	10 β
3.64	1	dd, J=6.7 & 15.8	10 α
2.90	1	b	11
2.75 - 2.78	1	m	17 β
2.55 - 2.59	1	m	17 α
2.29 - 2.33	1	m	9
1.21 - 2.15	11	m	7, 8, 12, 13, 14, 15

Legend: d=doublet, dd=double doublet, m=multiplet, b=broad

See Appendix II for the original ^1H NMR spectrum of alkaloid #1.

The signals at δ 4.24 and δ 3.64 could be assigned to H-10 β and H-10 α , respectively, because of the larger geminal coupling constant (15.8 Hz) and the lower chemical shifts [58], together with the downfield signals at δ 5.94, δ 6.42 and δ 7.25 due to the 2-pyridone ring.

Irradiation at δ 2.31 (centred, H-9) caused the double doublet at δ 3.64 due to H-10 α to become a doublet (J = 15.8 Hz, vicinal coupling depleted). Concurrently, upon irradiation at δ 3.64 (H-10 α), the multiplet at δ 2.31 was changed into sharper peaks. This observation gave evidence that H-9 and H-10 are vicinal to each other.

Irradiation at δ 4.24 (H-10 β) caused the double doublet at δ 3.64 due to H-10 α to become a doublet (J = 6.7, geminal coupling depleted). Conversely, the signal at δ 4.24 became a singlet when the signal at δ 3.64 was being irradiated. This observation provided evidence that H-10 α and H-10 β are geminal to each other.

The ^1H NMR spectrum (CDCl_3) of alkaloid #1 had no observable changes after D_2O was added, confirming that no exchangeable protons are present in the molecule.

The ^{13}C NMR signals of alkaloid #1 were assigned as shown in Table 16 by comparison with the spectra data of cytisine-type alkaloids [53,59].

Table 16. ^{13}C NMR chemical shifts of alkaloid #1 in CDCl_3 .

Chemical shifts (δ , ppm)	Assignment (carbon no.) see Figure 10
163.7	2
116.6	3
138.6	4
104.5	5
142.2	6
35.4	7
27.7	8
33.0	9
44.9	10
66.1	11
29.8	12
24.4	13
25.3	14
56.2	15
63.5	17

See Appendix III for the original ^{13}C NMR spectrum of alkaloid #1.

From these results, the structure of alkaloid #1 was presumed to be (-)-thermopsine as shown in Figure 10.

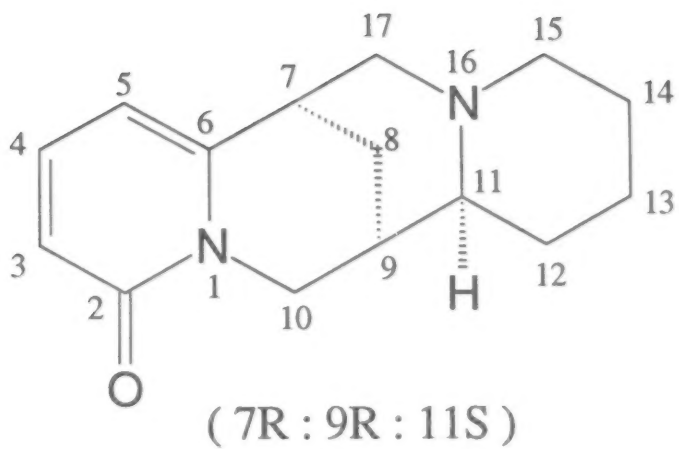


Figure 10. (-)-Thermopsine.

F.2. Alkaloid #2

The characteristic ^1H NMR signals of alkaloid #2 were assigned as shown in Table 17 from analysis of a D_2O exchange experiment, decoupling experiments [54] and comparison with the spectra of cytisine-type alkaloids [35,53,55-58].

Table 17. ^1H NMR chemical shifts for alkaloid #2 in CDCl_3 .

Chemical shifts (δ , ppm)	No. of protons	Multiplicity (J, Hz)	Assignment
7.25	1	dd, J=7.0 & 9.1	2-pyridone H
6.41	1	d, J=8.5	2-pyridone H
5.96	1	d, J=6.7	2-pyridone H
4.04	1	d, J=15.4	unknown
3.87	1	dd, J=6.6 & 15.4	unknown
3.35	1	dd, J=2.8 & 10.9	unknown
2.84 - 2.94	2	m	unknown
2.44	1	d, J=11.2	unknown

Legend: d=doublet, dd=double doublet, m=multiplet

See Appendix II for the original ^1H NMR spectrum of alkaloid #2.

There were signals observed in the range from δ 1.11 to δ 2.13 which appeared so compressed and poorly-resolved that peak assignments were not able to be made.

Irradiation at δ 4.04 caused the double doublet at δ 3.87 to become a doublet ($J = 6.6$ Hz, geminal coupling depleted). On the other hand, the signal at δ 4.04 became a singlet when the signal at δ 3.87 was being irradiated. This observation provided evidence that the protons due to these two signals are vicinal to each other.

The ^1H NMR spectrum (CDCl_3) of alkaloid #2 had no observable changes after D_2O was added, confirming that no exchangeable protons are present in the molecule.

The ^{13}C NMR signals of alkaloid #2 were assigned as shown in Table 18.

Table 18. ^{13}C NMR chemical shifts of alkaloid #2 in CDCl_3 .

Chemical shifts (δ , ppm)	Assignment
163.5	2-pyridone carbon
152.0	2-pyridone carbon
138.6	2-pyridone carbon
116.5	2-pyridone carbon
104.5	2-pyridone carbon
63.0	unknown
54.3	unknown
52.9	unknown
51.5	unknown
35.5	unknown
32.5	unknown
29.2	unknown
25.5	unknown
22.5	unknown
20.7	unknown
19.1	unknown

See Appendix III for the original ^{13}C NMR spectrum of alkaloid #2.

By comparing the spectrum data of alkaloid #2 with other known cytisine-type alkaloids, alkaloid #2 was presumed to be a lupine-type alkaloid which contains a 2-pyridone ring.

F.3. Alkaloid #3

All the ^1H NMR signals of alkaloid #3 were assigned as shown in Table 19 from analysis of a D_2O exchange experiment, a decoupling experiment [54] and comparison with the spectra data of cytosine-type alkaloids [35,53,55-58].

Table 19. ^1H NMR chemical shifts for alkaloid #3 in CDCl_3 .

Chemical shifts (δ , ppm)	No. of protons	Multiplicity (J, Hz)	Assignment (carbon no.) see Figure 11
7.27	1	dd, J=7.3 & 8.5	4
6.43	1	d, J=8.9	3
5.98	1	d, J=5.8	5
4.05	1	d, J=15.4	10B
3.89	1	dd, J=6.6 & 15.4	10 α
2.44	1	b	9
2.83 - 2.94	3	m	7, 11 α , 13 α
2.23 - 2.30	2	m	11B, 13B
2.14	3	s	14
1.72 - 1.88	2	m	8

Legend: d=doublet, dd=double doublet, m=multiplet, b=broad, s=singlet

See Appendix II for the original ^1H NMR spectrum of alkaloid #3.

The chemical shifts at δ 4.05 and δ 3.89 could be assigned to H-10 β and H-10 α , respectively, because of the geminal coupling constant (15.4 Hz) and the lower chemical shifts [58], together with the downfield signals at δ 5.98, δ 6.43 and δ 7.27 due to the 2-pyridone ring.

H-11 β and H-13 β are more shielded than the corresponding α protons because of the shielding effect of the lone pair electrons on nitrogen and the presence of an equatorial methyl group.

Irradiation at δ 2.44 (H-9) caused the double doublet at δ 3.89 due to H-10 α to become a doublet (J = 15.4 Hz, vicinal coupling depleted). On the other hand, on irradiation of δ 3.89 (H-10 α), the broad peak at δ 2.44 became sharper. It indicated that H-9 and H-10 are vicinal to each other.

Irradiation at δ 4.05 (H-10 β) converted the double doublet at δ 3.89 due to H-10 α to a doublet (J = 6.6 Hz, geminal coupling depleted). In addition, the signal at δ 4.05 became a singlet when the signal at δ 3.89 was being irradiated. It confirmed that H-10 α and H-10 β are geminal to each other.

The ^1H NMR spectrum (CDCl_3) of alkaloid #3 has no observable changes after D_2O was added, confirming that no exchangeable protons are present in the molecule.

The ^{13}C NMR signals of alkaloid #3 were assigned as shown in Table 20 by comparison with the spectra data of cytosine-type alkaloids [53,59].

Table 20. ^{13}C NMR chemical shifts of alkaloid #3 in CDCl_3 .

Chemical shifts (δ , ppm)	Assignment (carbon no.) see Figure 11
163.7	2
116.9	3
138.6	4
104.6	5
142.2	6
35.3	7
25.3	8
27.9	9
49.8	10
62.0	11
62.3	13
46.1	14

See Appendix III for the original ^{13}C NMR spectrum of alkaloid #3.

From the results obtained, the structure of alkaloid #3 was presumed to be (-)-N-methylcytisine, as shown in Figure 11.

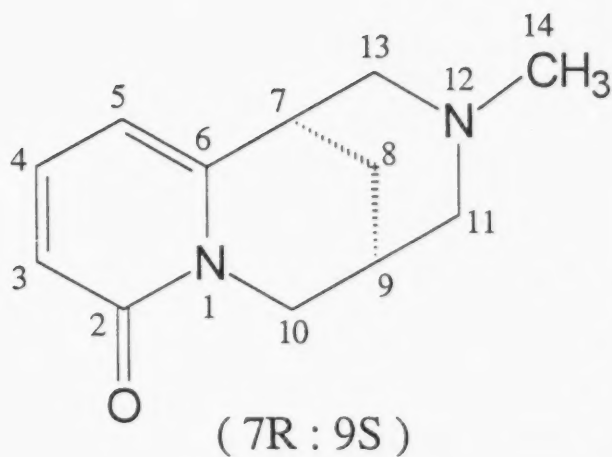


Figure 11. (-)-N-methylcytisine.

F.4. Alkaloid #4

The characteristic ^1H NMR signals of alkaloid #4 were assigned as shown in Table 21 from analysis of a D_2O exchange experiment, decoupling experiments [54] and comparison with the spectra of cytisine-type alkaloids [35,53,55-58].

Table 21. ^1H NMR chemical shifts for alkaloid #4 in CDCl_3 .

Chemical shifts (δ , ppm)	No. of protons	Multiplicity (J, Hz)	Assignment
7.25	1	dd, J=6.9 & 9.0	2-pyridone H
6.41	1	d, J=8.7	2-pyridone H
5.95	1	d, J=6.8	2-pyridone H

Legend: d=doublet, dd=double doublet

See Appendix III for the original ^{13}C NMR spectrum of alkaloid #4.

There were signals observed in the range from δ 1.10 to δ 4.29 which appeared so compressed and poorly-resolved that peak assignments were not able to be made.

The ^1H NMR spectrum (CDCl_3) of alkaloid #4 has no observable changes after D_2O was added, confirming that no exchangeable protons are present in the molecule.

The ^{13}C NMR signals of alkaloid #4 were assigned as shown in Table 22.

Table 22. ^{13}C NMR chemical shifts of alkaloid #4 in CDCl_3 .

Chemical shifts (δ , ppm)	Assignment
163.6	2-pyridone carbon
151.6	2-pyridone carbon
138.6	2-pyridone carbon
116.7	2-pyridone carbon
104.5	2-pyridone carbon
88.9	unknown
66.7	unknown
54.4	unknown
33.9	unknown
29.8	unknown
25.8	unknown
23.2	unknown
19.9	unknown
16.7	unknown

See Appendix III for the original ^{13}C NMR spectrum of alkaloid #4. By comparing the spectrum data of alkaloid #4 with other known cytisine-type alkaloids, alkaloid #4 was presumed to be a lupine-type alkaloid which contains an 2-pyridone ring.

F.5. Alkaloid #5

All the ^1H NMR signals of alkaloid #5 were assigned as shown in Table 23 from analysis of a D_2O exchange experiment, a decoupling experiment [54] and comparison with the spectra data of cytosine and cytosine-type alkaloids [35,53,55-58].

Table 23. ^1H NMR chemical shifts for alkaloid #5 in CDCl_3 .

Chemical shifts (δ , ppm)	No. of protons	Multiplicity (J, Hz)	Assignment (carbon no.) see Figure 12
7.30	1	dd, J=7.0 & 9.1	4
6.46	1	d, J=9.1	3
6.00	1	d, J=6.8	5
4.13	1	d, J=15.6	10 β
3.90	1	dd, J=6.6 & 15.5	10 α
2.90 - 3.13	5	m	7, 11, 13
2.33	1	b	9
1.95	2	m	8
1.57	1	b	N-H

Legend: d=doublet, dd=double doublet, m=multiplet, b=broad

See Appendix II for the original ^1H NMR spectrum of alkaloid #5.

The chemical shifts at δ 4.13 and δ 3.90 could be assigned to H-10 β and H-10 α respectively because of the larger geminal coupling constant (15.6 Hz) and the lower chemical shifts [58], together with the downfield signals at δ 6.00, δ 6.46 and δ 7.30 due to the 2-pyridone ring.

When the signal at δ 2.33 (H-9) was being irradiated, the double doublet at δ 3.90 (H-10 α) was converted into a doublet (J = 15.6 Hz, vicinal coupling depleted). In addition, the signal at δ 2.33 became sharper when the signal at δ 3.90 was being irradiated. This observation suggested that H-9 and H-10 are vicinal to each other.

After D_2O was added, the signal at δ 1.57 disappeared, which confirmed that the peak at δ 1.57 is due to exchangeable proton. The highly shielded protons at δ 1.95 must be distant from the electronegative element or exempted from the deshielding effect of magnetic anisotropy. The ^1H NMR spectrum (CDCl_3) of alkaloid #5 was superimposable on that of the authentic sample of (-)-cytisine.

The ^{13}C NMR signals of alkaloid #5 were assigned as shown in Table 24 by comparison with the spectra data of cytisine-type alkaloids [53,59].

Table 24. ^{13}C NMR chemical shifts of alkaloid #5 in CDCl_3 .

Chemical shifts (δ , ppm)	Assignment (carbon no.) see Figure 12
163.7	2
116.8	3
138.8	4
105.0	5
150.8	6
35.4	7
26.2	8
27.6	9
49.6	10
52.7	11
53.7	13

See Appendix III for the original ^{13}C NMR spectrum of alkaloid #5.

The ^{13}C NMR spectrum (CDCl_3) of alkaloid #5 was superimposable on that of the authentic sample of (-)-cytisine.

After considering all the above information, the structure of alkaloid #5 was presumed to be (-)-cytisine, as shown in Figure 12.

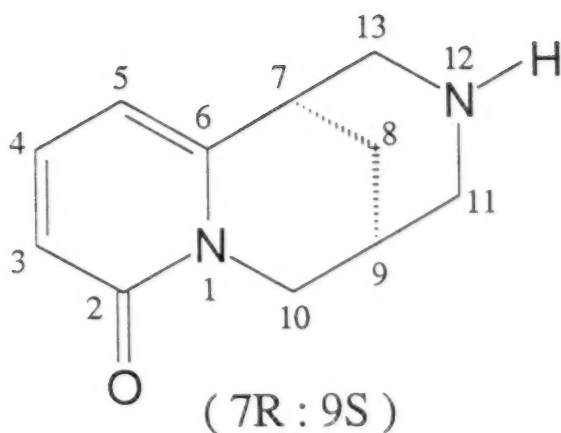


Figure 12. (-)-Cytisine.

G. Ultraviolet spectroscopy analysis

Table 25. UV absorption peaks for the isolated alkaloids of Thermopsis rhombifolia from E-TrI.

Sample	$\lambda_{\text{max}}^{\text{H}_2\text{O}}$
Alkaloid #1	231.7 nm (log ϵ = 3.80) 305.0 nm (log ϵ = 3.90)
Alkaloid #2	231.8 nm 305.6 nm
Alkaloid #3	231.5 nm (log ϵ = 3.80) 305.1 nm (log ϵ = 3.89)
Alkaloid #4	231.7 nm 304.8 nm
Alkaloid #5	232.3 nm (log ϵ = 3.80) 306.4 nm (log ϵ = 3.90)
(-)-Cytisine	232.3 nm (log ϵ = 3.80) 306.4 nm (log ϵ = 3.90)

See Appendix IV for the original UV spectra of each isolated alkaloid.

The presumed molecular structures of the isolated alkaloids all have the following characteristic chromophore. See Figure 13.

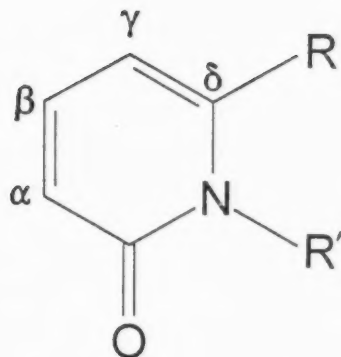


Figure 13. Chromophore for the isolated alkaloids in Thermopsis rhombifolia.

By using Woodward's rules [60] for the ultraviolet absorption of enones (applicable for electron transfer transition : $\pi \longrightarrow \pi^*$ transition), the $\lambda_{\max}$ for the chromophore as shown in Figure 13 was calculated in Table 26.

Table 26. Calculation on the $\lambda_{\max}$ for the electron transfer transition of the isolated alkaloids of Thermopsis rhombifolia from E-TrI.

Characteristic	Shift in $\lambda_{\max}$
<u>Base value</u>	
Six-membered enone	+ 215 nm
<u>Increments for</u>	
Double bond extending conjugation	+ 30 nm
Homocyclic diene	+ 39 nm
δ -ring residue	+ 18 nm
Exocyclic double bond	+ 5 nm
Total ($\lambda_{\max}$)	= 307 nm

One of the experimental UV absorption peaks of all the isolated alkaloids conform to this calculated UV absorption ($\pi \longrightarrow \pi^*$ transition) that corresponds to the chromophore presumed above. Moreover, the UV spectra of the isolated alkaloids revealed absorption peaks at ca. 232 nm ($\log \epsilon = 3.80$) and ca. 305 nm ($\log \epsilon = 3.90$), indicating the presence of an 2-pyridone ring in the molecule [61]. The UV spectrum of alkaloid #5 was superimposable on that of the authentic sample of (-)-cytisine.

H. High Performance Liquid Chromatography analysis

The chromatographic patterns of the alkaloids in Thermopsis rhombifolia are shown in Figures 14-15.

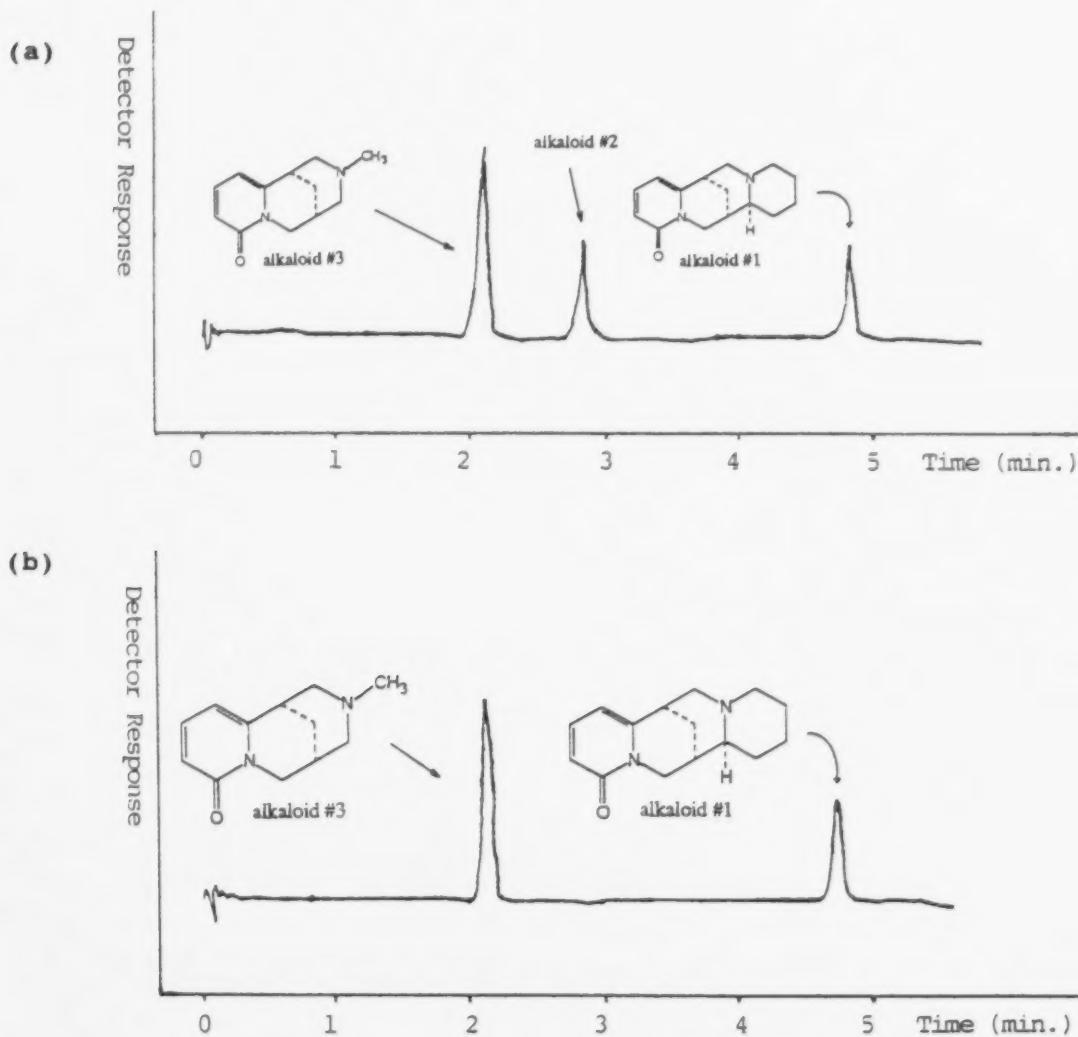


Figure 14. HPLC profile of the less polar group alkaloids from the crude extracts, E-TrI (a), E-TrII (b), and E-TrIII (c); HPLC analyses were carried out according to Table 5.

(c)

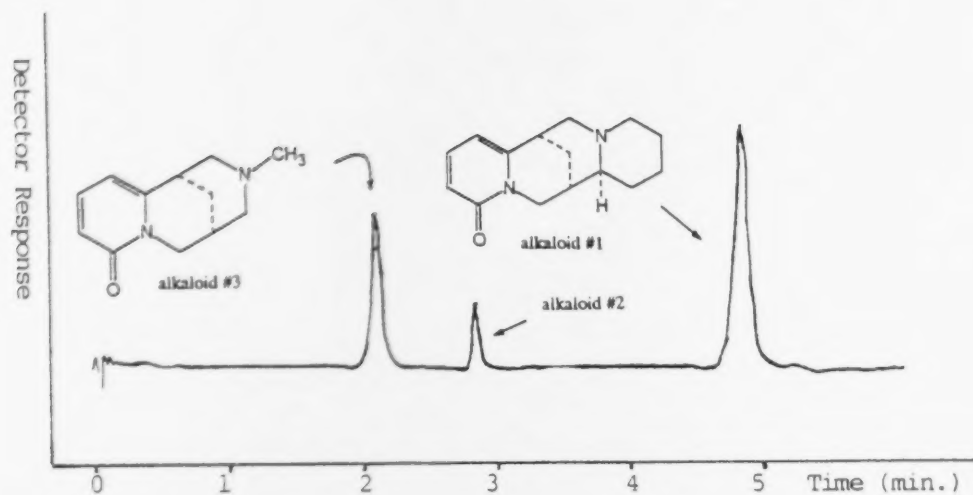


Figure 14. (continued)

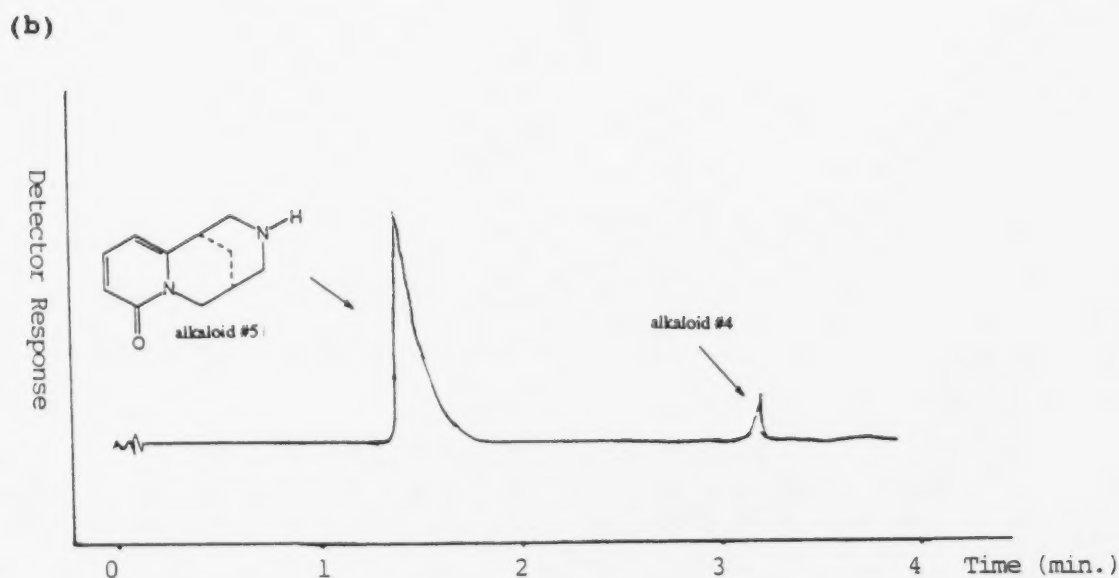
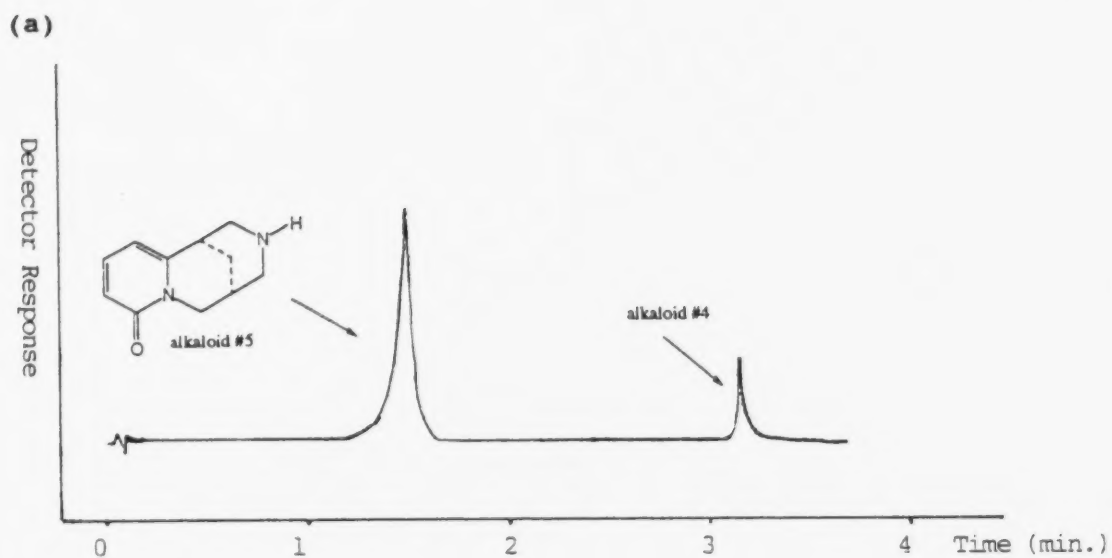


Figure 15. HPLC profile of the more polar group alkaloids from the crude extracts, E-TrI (a), E-TrII (b), and E-TrIII (c); HPLC analyses were carried out according to Table 5.

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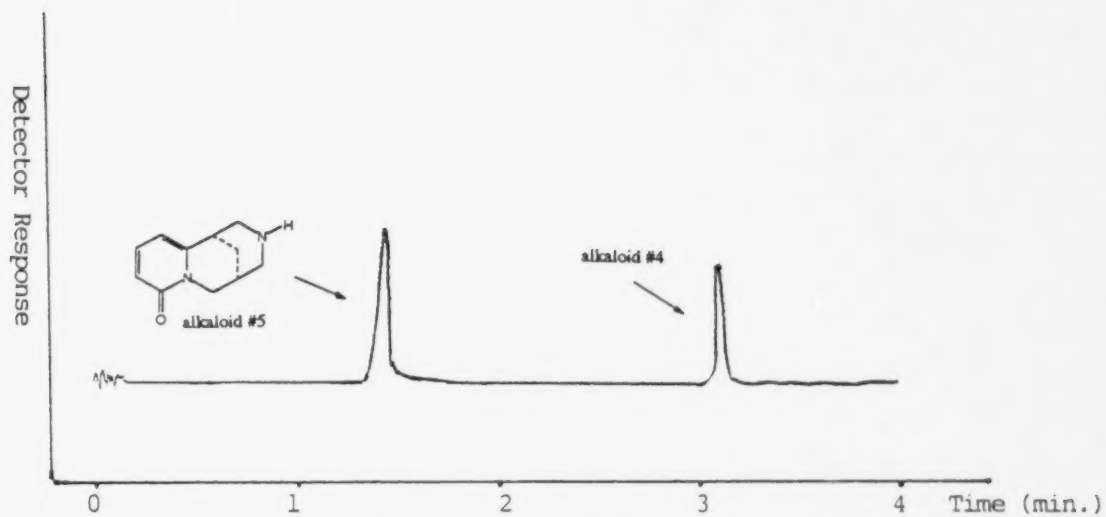


Figure 15. (continued)

Calibration curve data and the HPLC response data of the plant crude extracts for alkaloid #1 are shown in Table 27.

Table 27. Quantitative analysis of alkaloid #1 in the plant crude extracts.

Sample	Peak area	Retention time (min.)
Alkaloid #1 (5 ppm)	419740	4.81
Alkaloid #1 (10 ppm)	799890	4.80
Alkaloid #1 (15 ppm)	1150800	4.79
Alkaloid #1 (20 ppm)	1556000	4.79
Alkaloid #1 (30 ppm)	2400010	4.78
E-TrI (10-fold dilution)	1249100	4.79
E-TrII (5-fold dilution)	1319600	4.79
E-TrIII (75-fold dilution)	751270	4.81

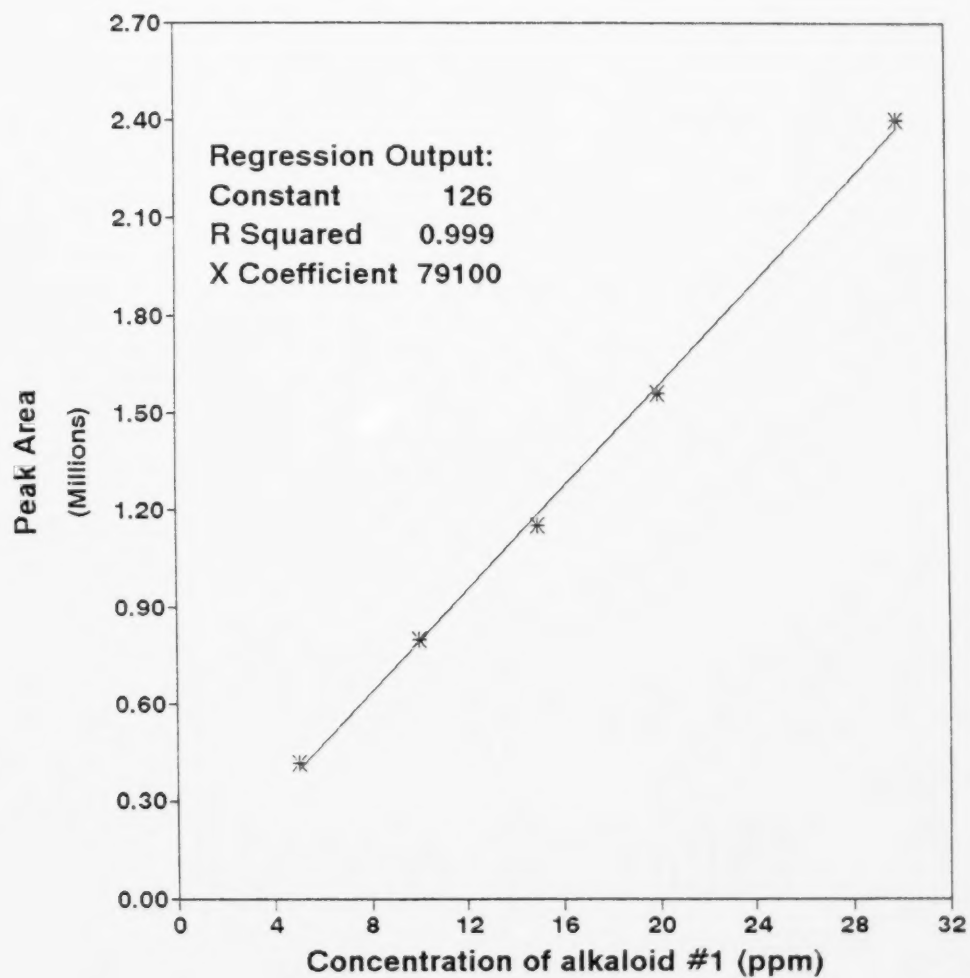


Figure 16. Calibration curve for alkaloid #1,
(-)-thermopsine.
* represents experimental data
— represents regression line

Calibration curve data and the HPLC response data of the plant crude extracts for alkaloid #3 are shown in Table 28.

Table 28. Quantitative analysis of alkaloid #3 in the plant crude extracts.

Sample	Peak area	Retention time (min.)
Alkaloid #3 (5 ppm)	568030	2.06
Alkaloid #3 (8 ppm)	798790	2.06
Alkaloid #3 (10 ppm)	981260	2.06
Alkaloid #3 (15 ppm)	1415800	2.05
Alkaloid #3 (20 ppm)	1781500	2.06
E-TrI (20-fold dilution)	982750	2.05
E-TrII (10-fold dilution)	1525400	2.05
E-TrIII (75-fold dilution)	862520	2.05

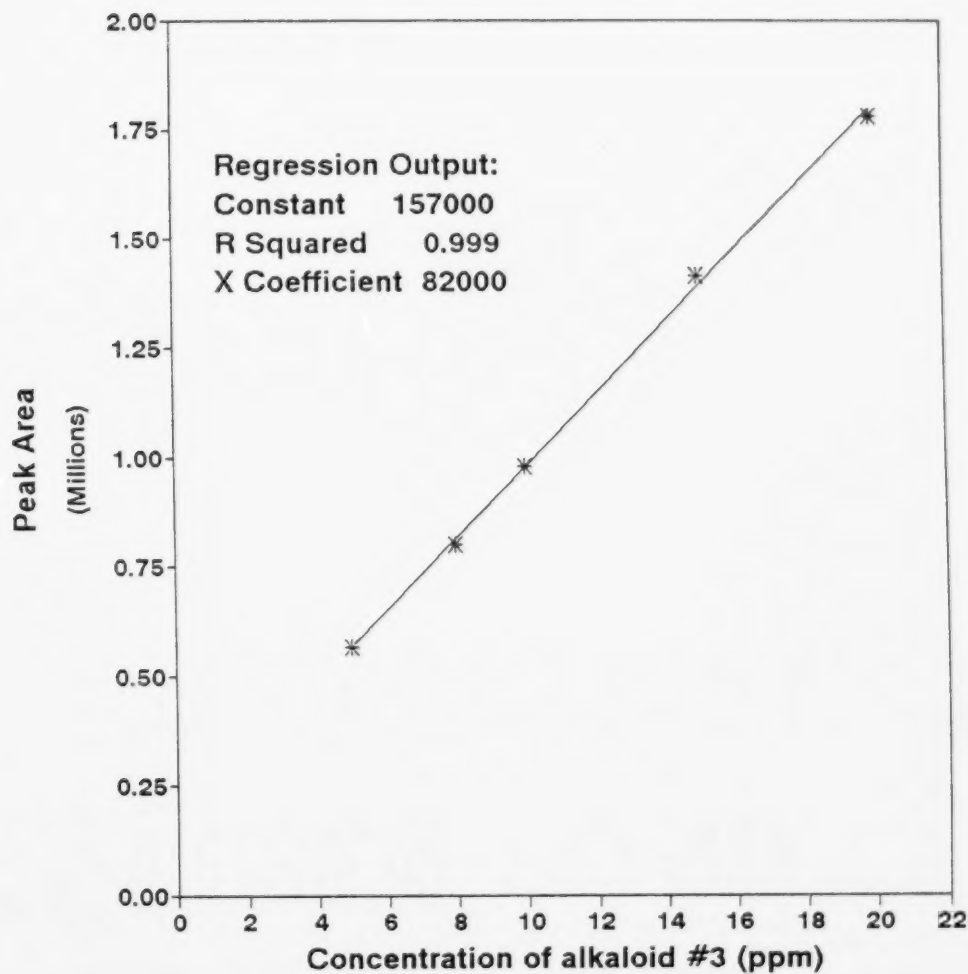


Figure 17. Calibration curve for alkaloid #3,
(-)-N-methylcytisine.
* represents experimental data
— represents regression line

Calibration curve data and the HPLC response data of the plant crude extracts for alkaloid #5 are shown in Table 29.

Table 29. Quantitative analysis of alkaloid #5 in the plant crude extracts.

Sample	Peak area	Retention time (min.)
Alkaloid #5 (3 ppm)	403465	1.48
Alkaloid #5 (5 ppm)	634180	1.48
Alkaloid #5 (8 ppm)	933800	1.49
Alkaloid #5 (10 ppm)	1183600	1.48
Alkaloid #5 (15 ppm)	1751850	1.49
E-TrI (10-fold dilution)	1594200	1.48
E-TrII (40-fold dilution)	1115500	1.49
E-TrIII (20-fold dilution)	1505200	1.49

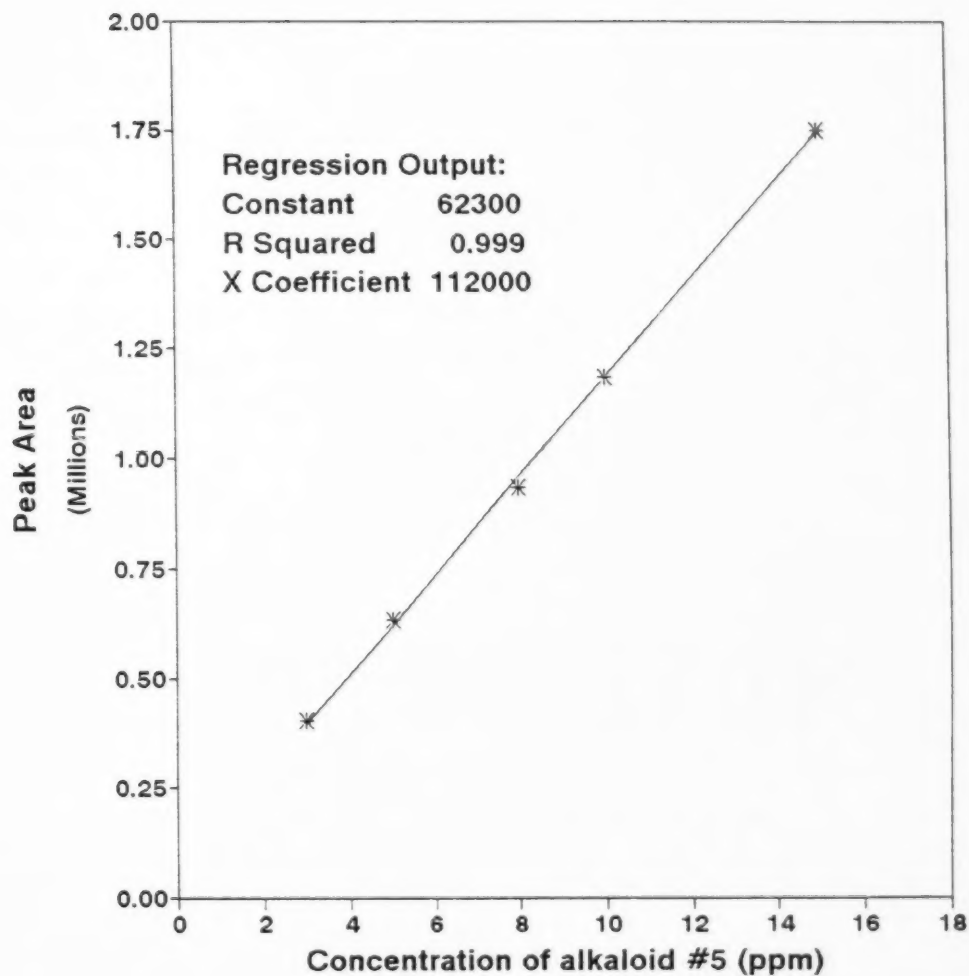


Figure 18. Calibration curve for alkaloid #5, (-)-cytisine.
* represents experimental data
— represents regression line

Although reference samples for alkaloids #2 and #4 were not available, they were confirmed to be quinolizidine alkaloids with a 2-pyridone ring which gave the same UV detector response as the other purified alkaloids. Consequently, the quantity of these two unknown alkaloids could be estimated by an area normalization method (assumption: alkaloids isolated from Thermopsis rhombifolia have the same detector response to the UV detector used in the HPLC method).

The HPLC response data for alkaloid #2 are shown in Table 30.

Table 30. Quantitative analysis of alkaloid #2 in the plant crude extracts.

Sample	Peak area	Retention time (min.)
TrI (10-fold dilution)	1332600	2.80
TrII (5-fold dilution)	not detected	not detected
TrIII (75-fold dilution)	286840	2.84

The HPLC response data for alkaloid #4 are shown in Table 31.

Table 31. Quantitative analysis of alkaloid #4 in the plant crude extracts.

Sample	Peak area	Retention time (min.)
TrI (10-fold dilution)	568540	3.14
TrII (40-fold dilution)	57771	3.15
TrIII (20-fold dilution)	1177600	3.14

Variations in alkaloidal content at different stages of growth are shown in Table 32.

Table 32. Variations of alkaloidal content in the aerial parts of Thermopsis rhombifolia at different stages during growth.

Alkaloid	<u>Concentration</u>		
	TrI*	TrII**	TrIII**
#1	0.93	0.21	0.71
#2	0.99	not detected	0.27
#3	1.3	0.42	0.65
#4	0.29	0.049	0.20
#5	0.80	0.95	0.26
Total	4.3	1.6	2.1

* concentration in mg/g dry weight of the plant

** concentration in mg/g fresh weight of the plant

TrI : collected at Tofield of Alberta on July 14, 1991
(during mature seeds period)

TrII : collected at Killam of Alberta on May 29, 1993
(during flowering period)

TrIII : collected at Ponoka of Alberta on July 13, 1994
(during mature seeds period)

#1 : (-)-thermopsine

#3 : (-)-N-methylcytisine

#5 : (-)-cytisine

PART FIVE

SUMMARY AND CONCLUSIONS

Summary and conclusions:

1. The extraction method with maceration in 75% ethanol and partitioning between aqueous and dichloromethane layers as outlined in PART THREE (EXPERIMENTAL) was found to be effective in extracting the basic constituents from Thermopsis rhombifolia.
2. The basic extracts of Thermopsis rhombifolia were investigated and four distinct alkaloids were found during the flowering period while five distinct alkaloids were found during the mature seeds period.
3. An exhaustive TLC examination of the various fractions failed to disclose the presence of other bases in any detectable amounts.
4. Four of the five distinct alkaloids isolated during the mature seeds period have the same identity as the four distinct alkaloids isolated during the flowering period.
5. The UV, IR, ^1H NMR AND ^{13}C NMR spectroscopic data confirmed that the bases detected and isolated from Thermopsis rhombifolia are α -pyridone-type alkaloids.

6. With the experimental support of TLC, co-TLC, m.p., mixed m.p., specific rotation, UV, IR, ^1H NMR (together with a series of decoupling and D_2O exchange experiments) and ^{13}C NMR, three of the isolated alkaloids from both the flowering and the mature seeds periods were characterized as (-)-thermopsine, (-)-N-methylcytisine and (-)-cytisine.
7. The structural identification of alkaloids #2 and #4 failed because of degradation or the small amount of pure isolated alkaloid that was available.
8. The HPLC method developed in this research was found to be efficient for both qualitative and quantitative analysis of lupine alkaloids in the aerial parts of Thermopsis rhombifolia. This method may be useful for biochemical, physiological, and chemotaxonomic studies of these alkaloids.
9. By comparing the results which were based on one total alkaloidal extraction of samples TrII and TrIII, the yields of basic crude extract and total alkaloidal content were found to be slightly higher in the mature seeds period than in the flowering period. Therefore, it may be better to harvest this plant during the mature seeds period in order to collect higher amounts of the basic compounds. However,

the variations of alkaloidal content may also be affected by the location of the plant. It would be interesting to have more comprehensive study on the alkaloidal contents during various stages of growth with respect to the location of the plant in order to determine the best time for maximum alkaloidal yield.

PART SIX

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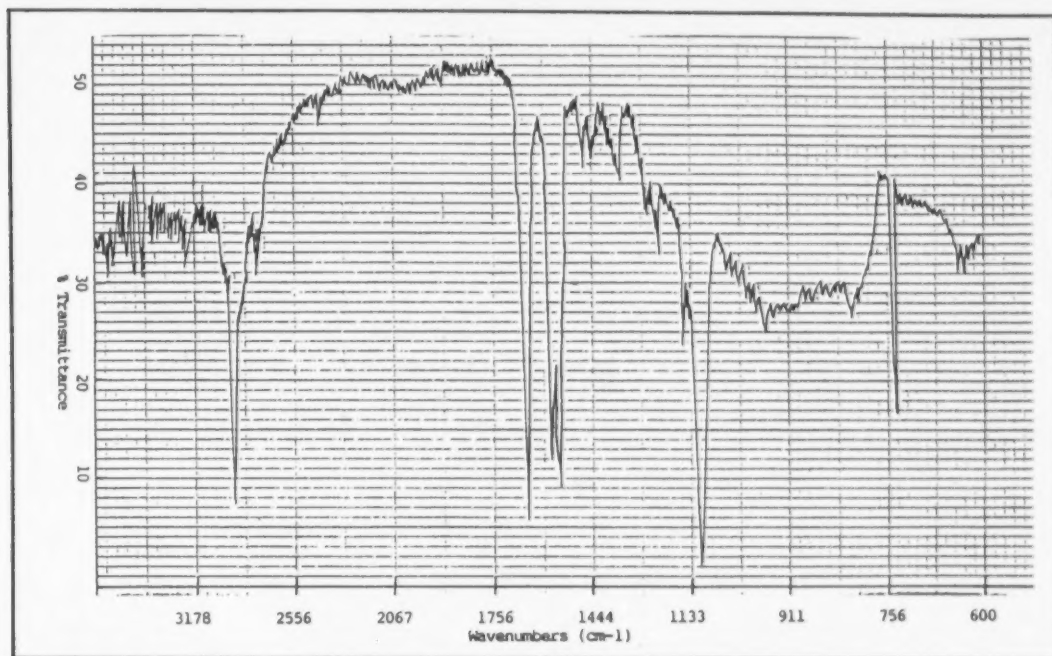
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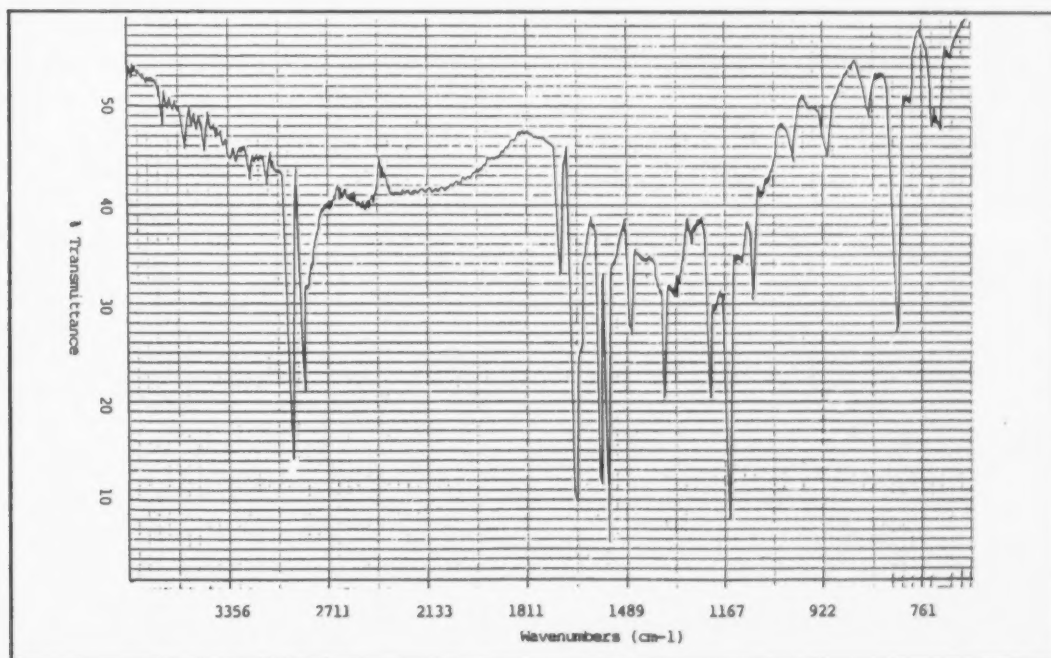
PART SEVEN

APPENDICES

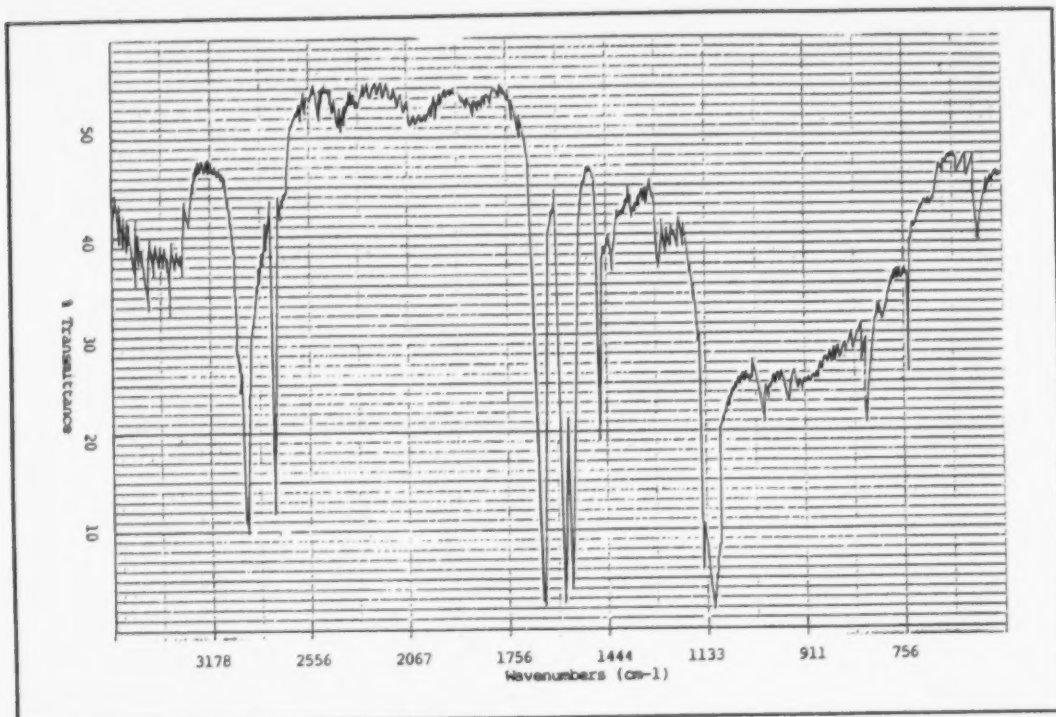
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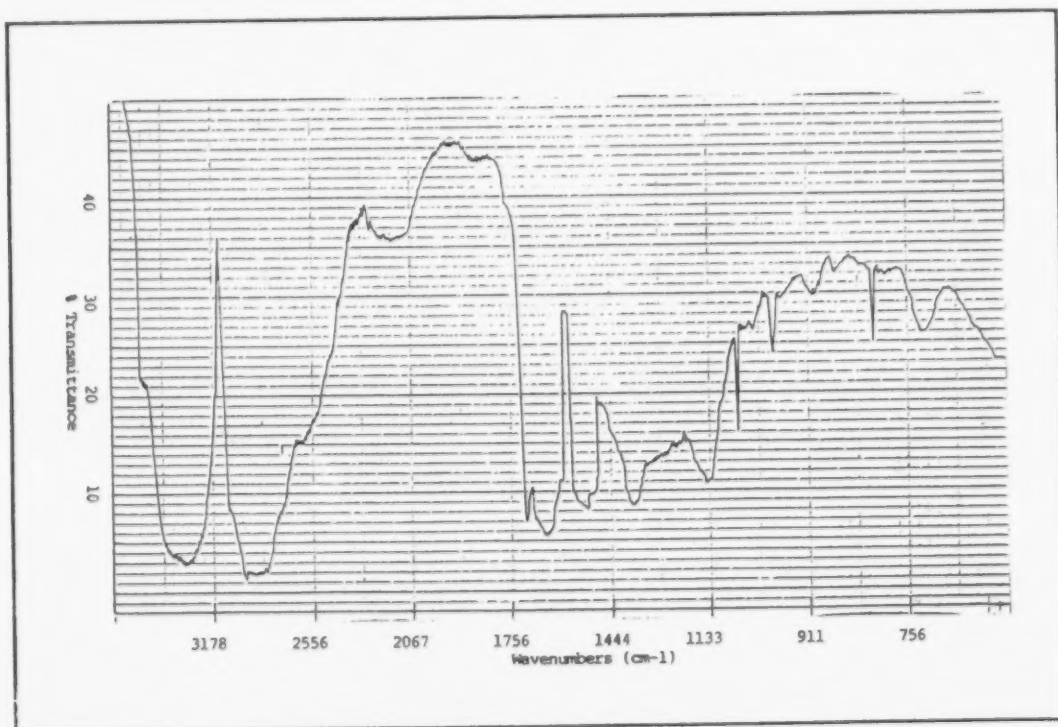
IR spectrum of alkaloid #1.



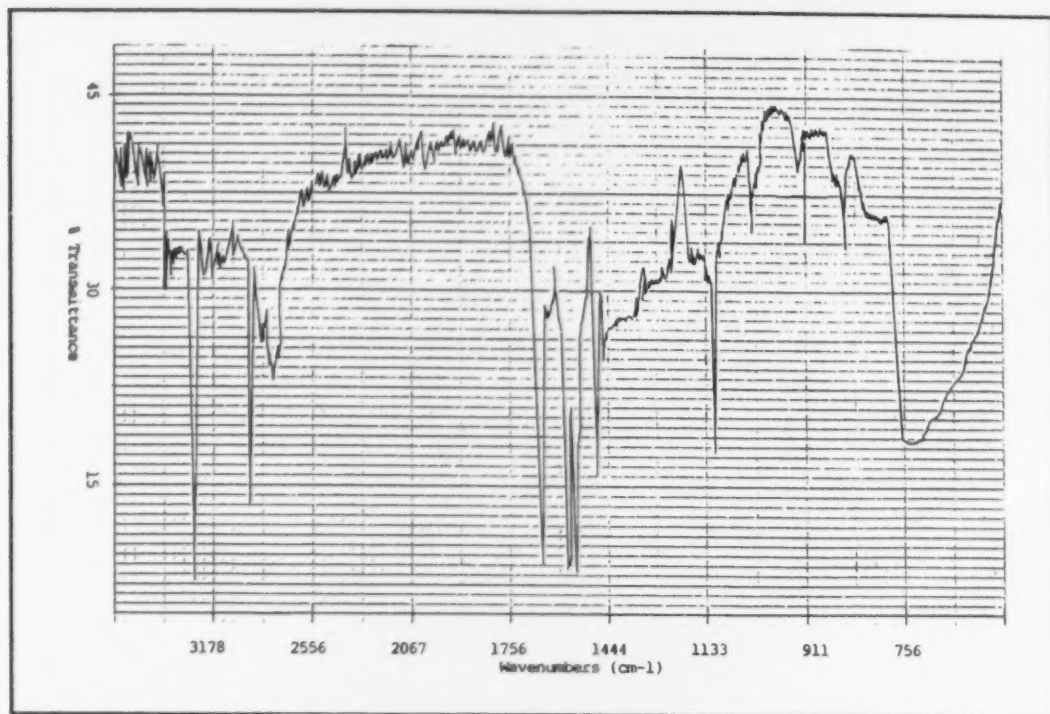
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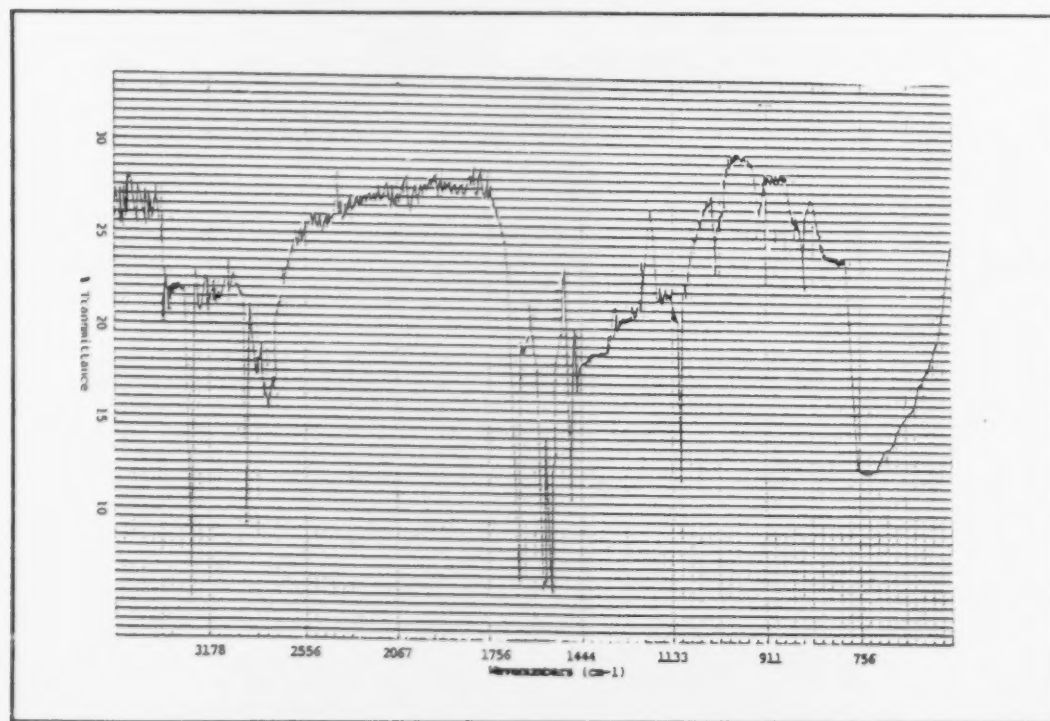
IR spectrum of alkaloid #3.



IR spectrum of alkaloid #4

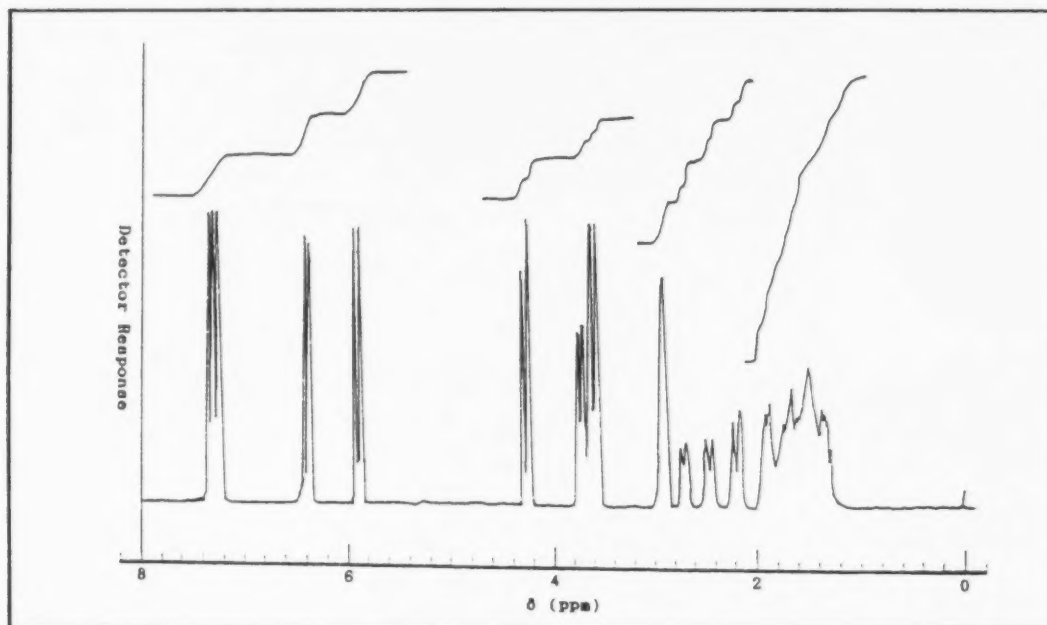


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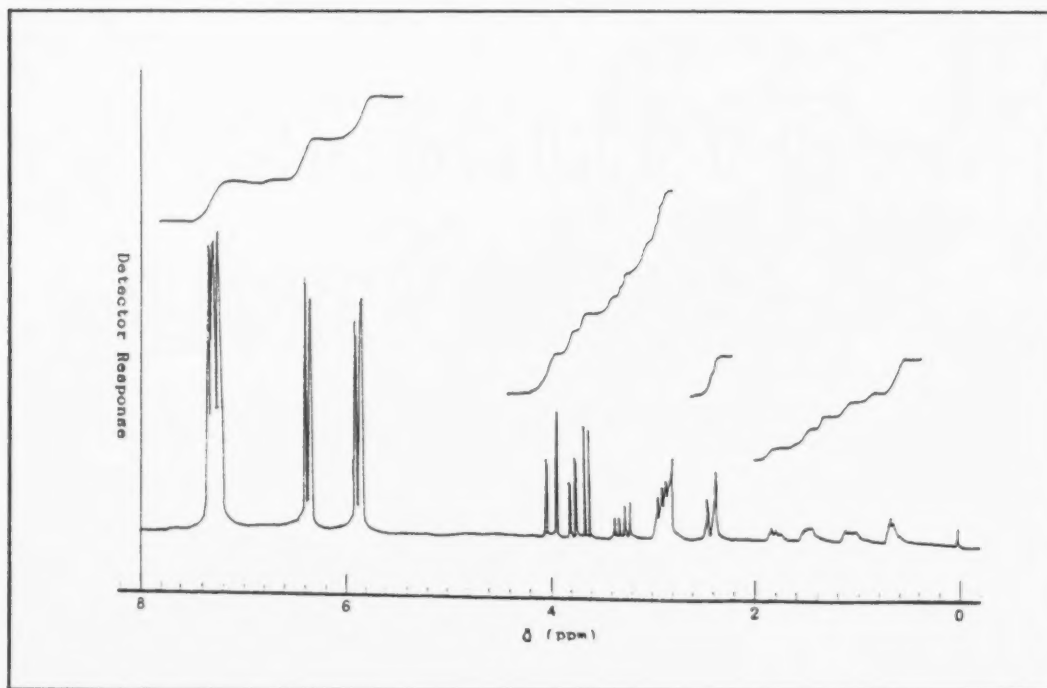


IR spectrum of (-)-cytisine.

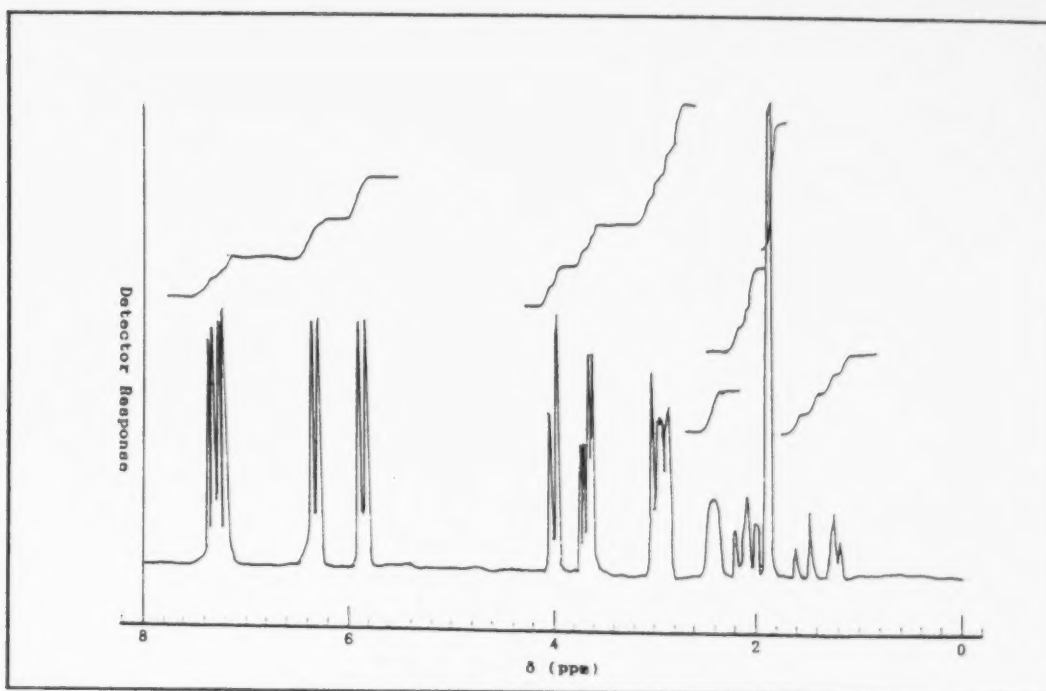
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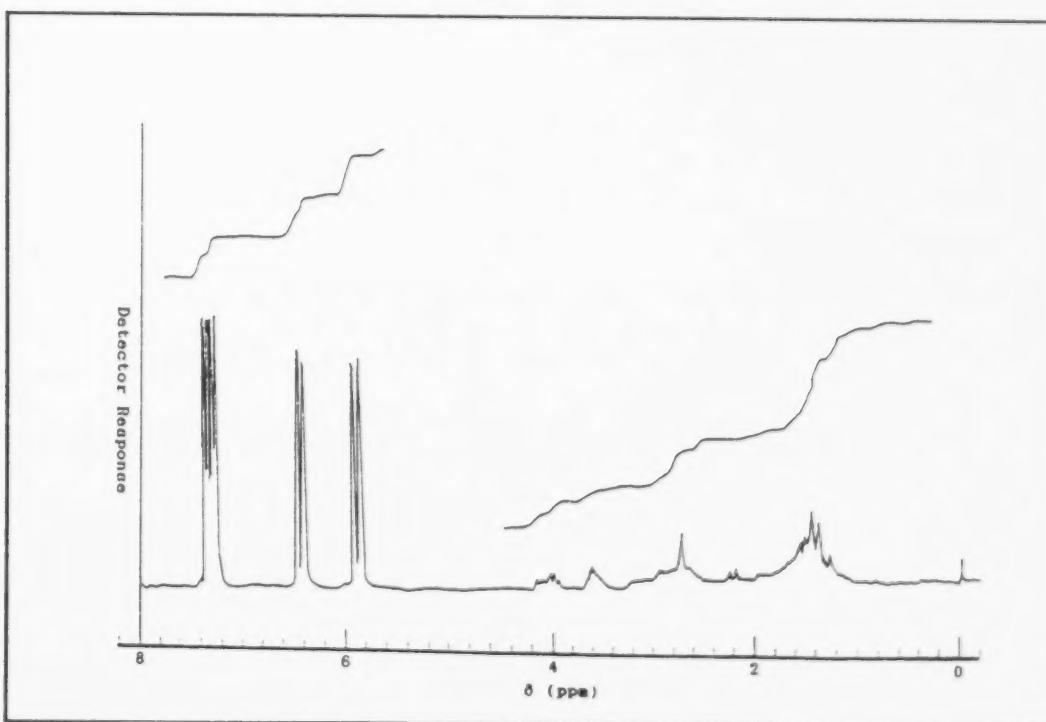
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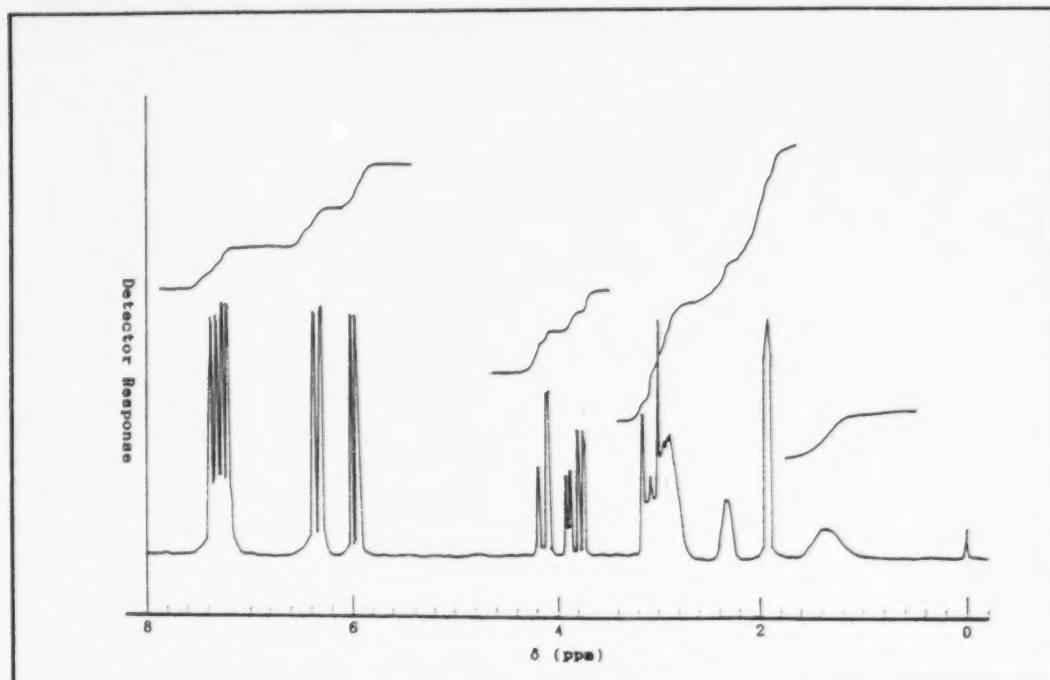
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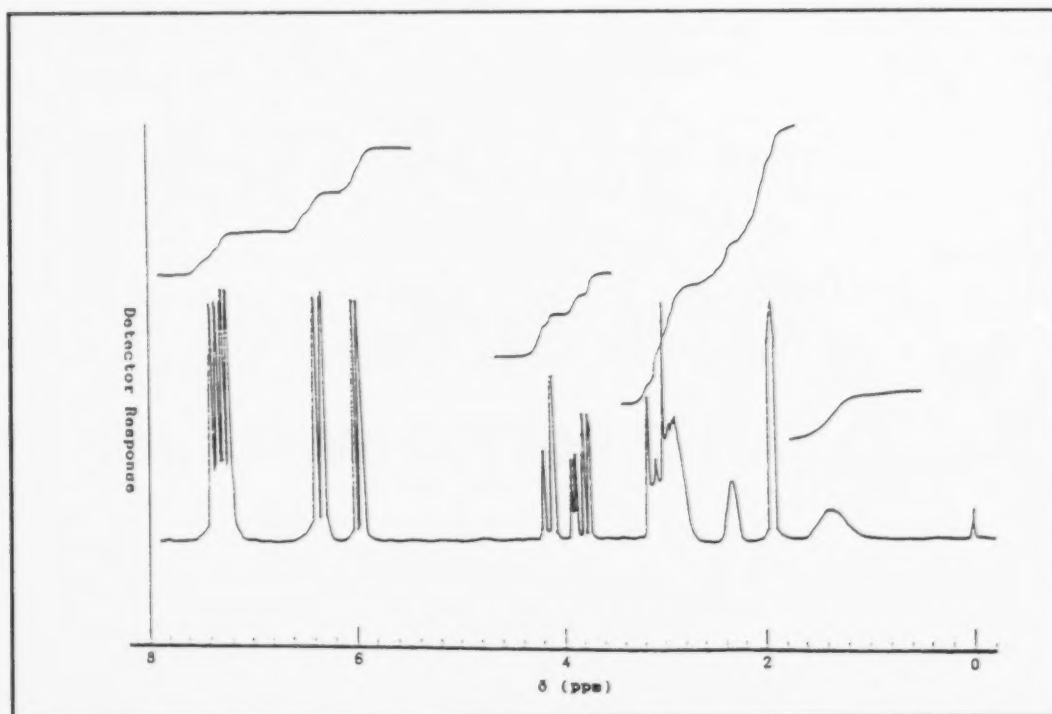
^1H NMR spectrum of alkaloid #3.



^1H NMR spectrum of alkaloid #4.

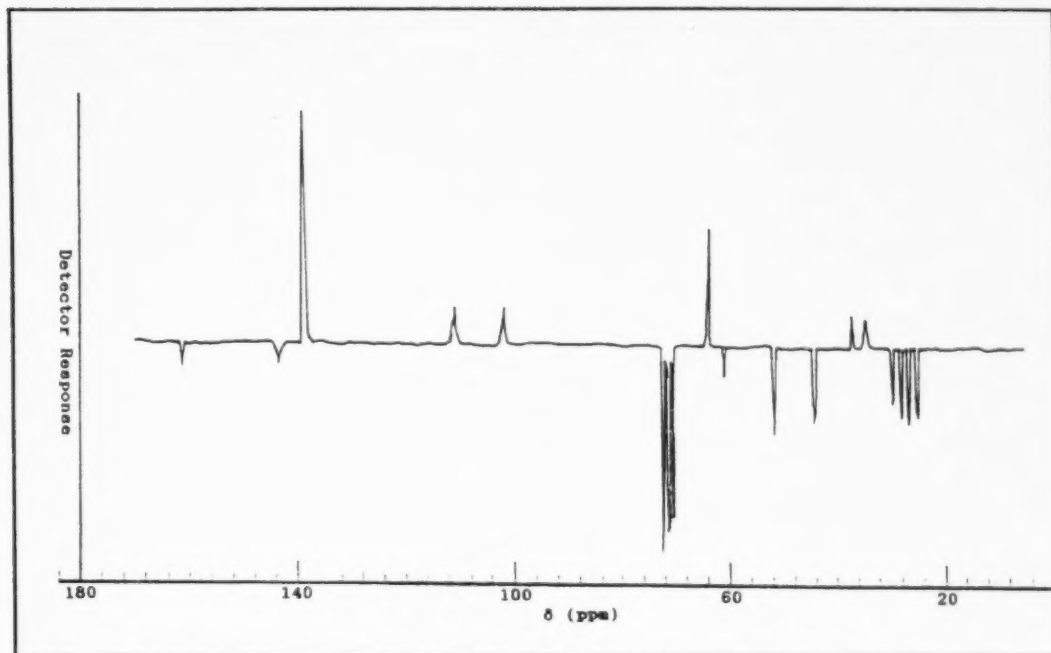


^1H NMR spectrum of alkaloid #5.

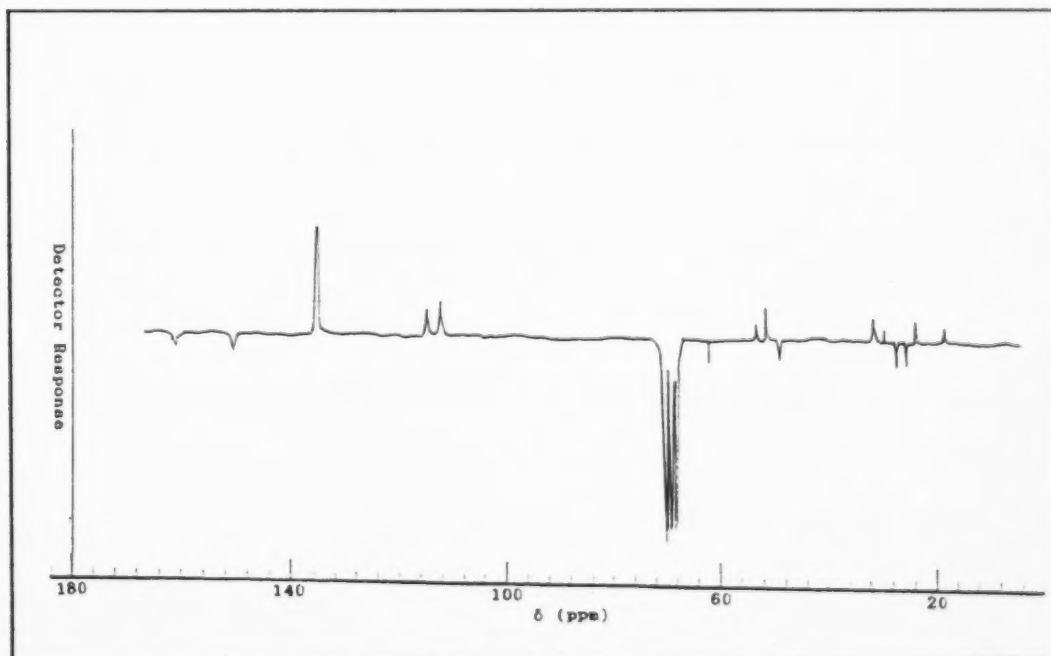


^1H NMR spectrum of (-)-cytisine.

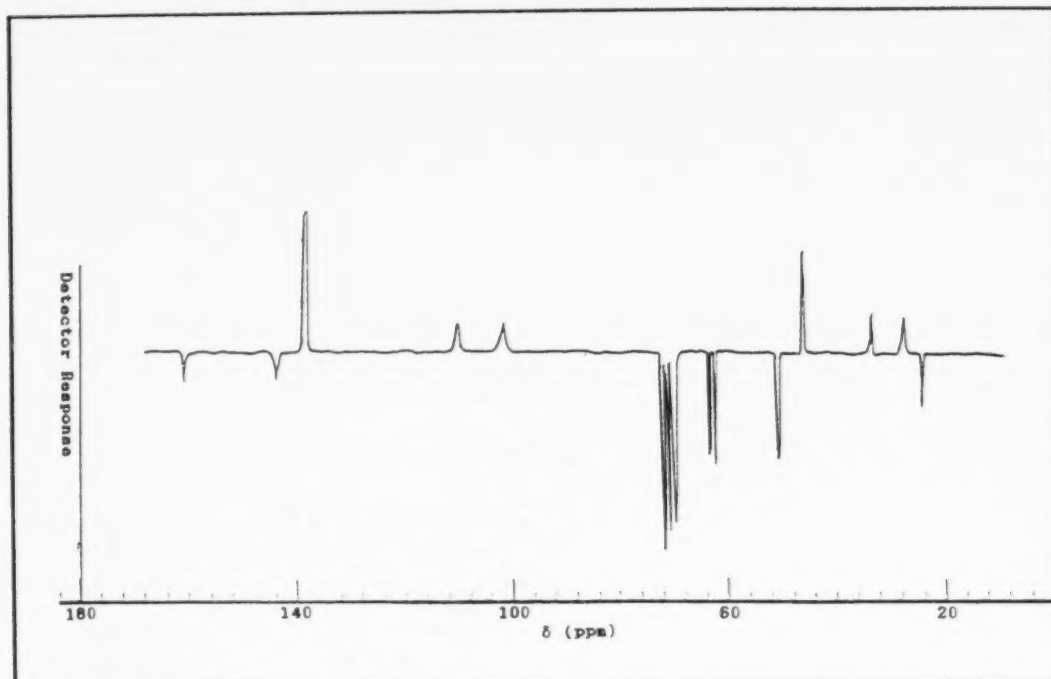
III. ^{13}C NMR spectra of the isolated alkaloids from Thermopsis rhombifolia



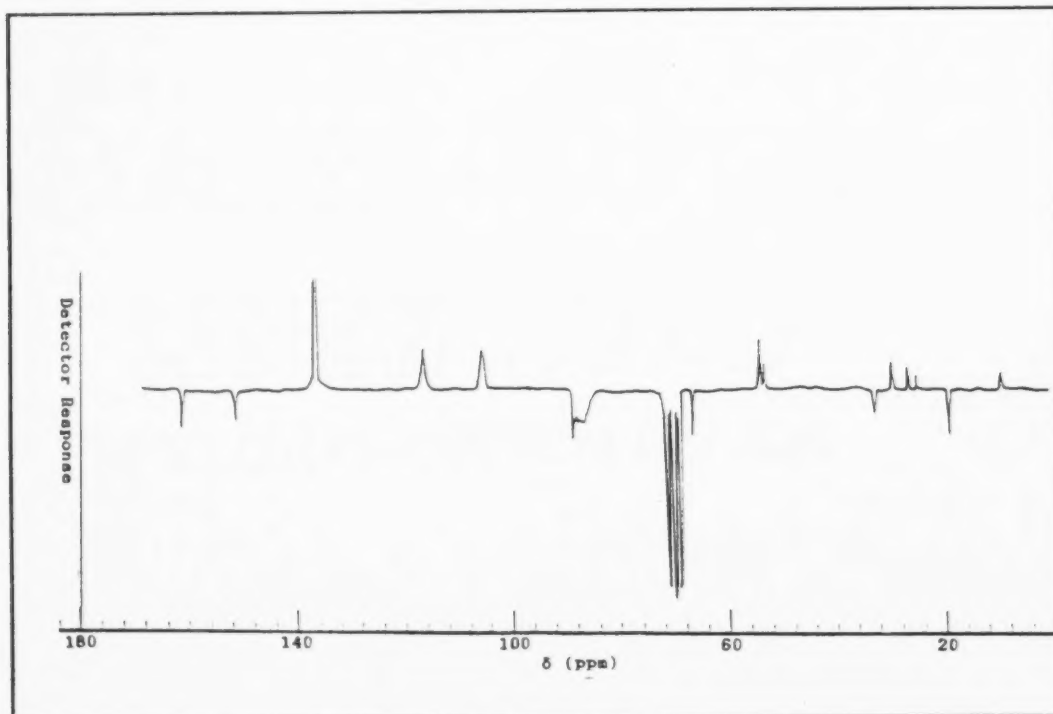
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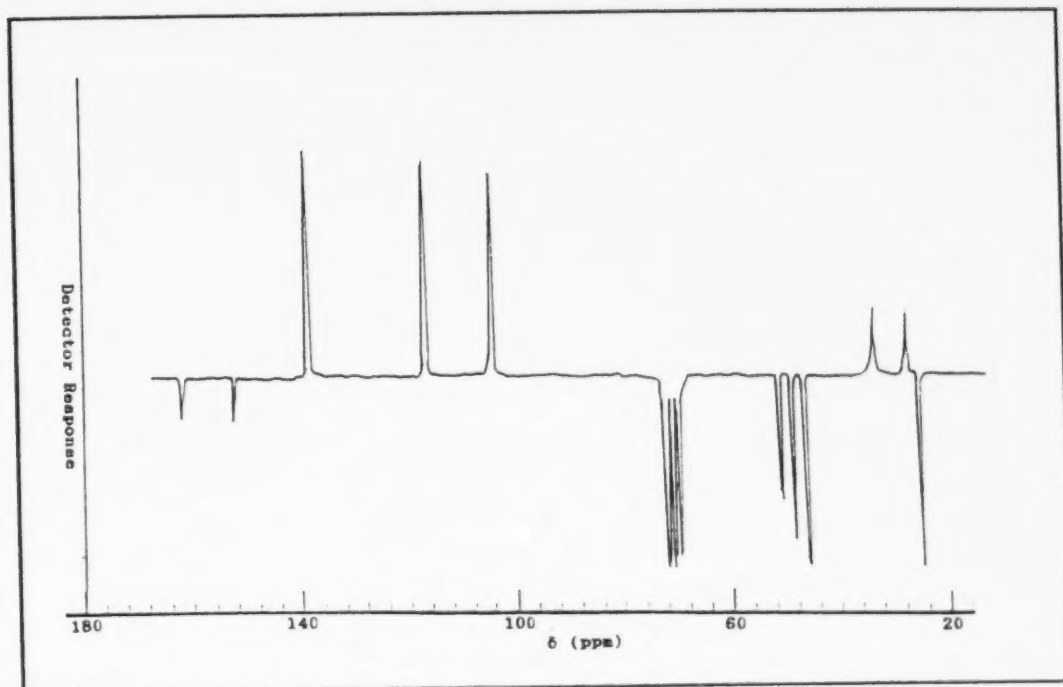
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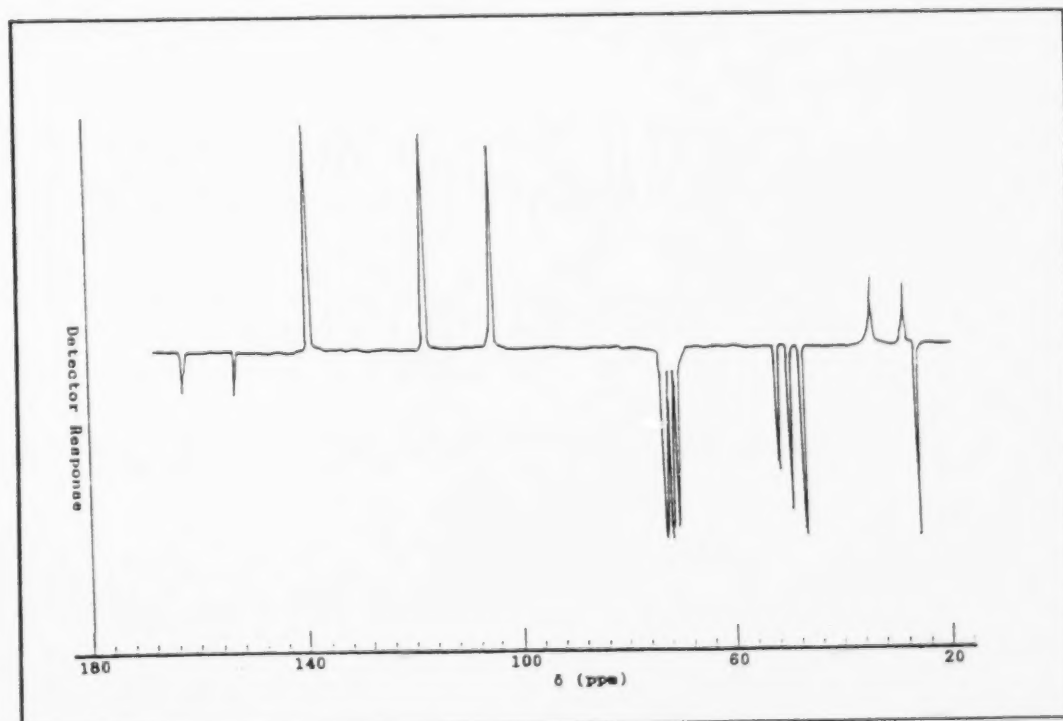
^{13}C NMR spectrum of alkaloid #3.



^{13}C NMR spectrum of alkaloid #4.

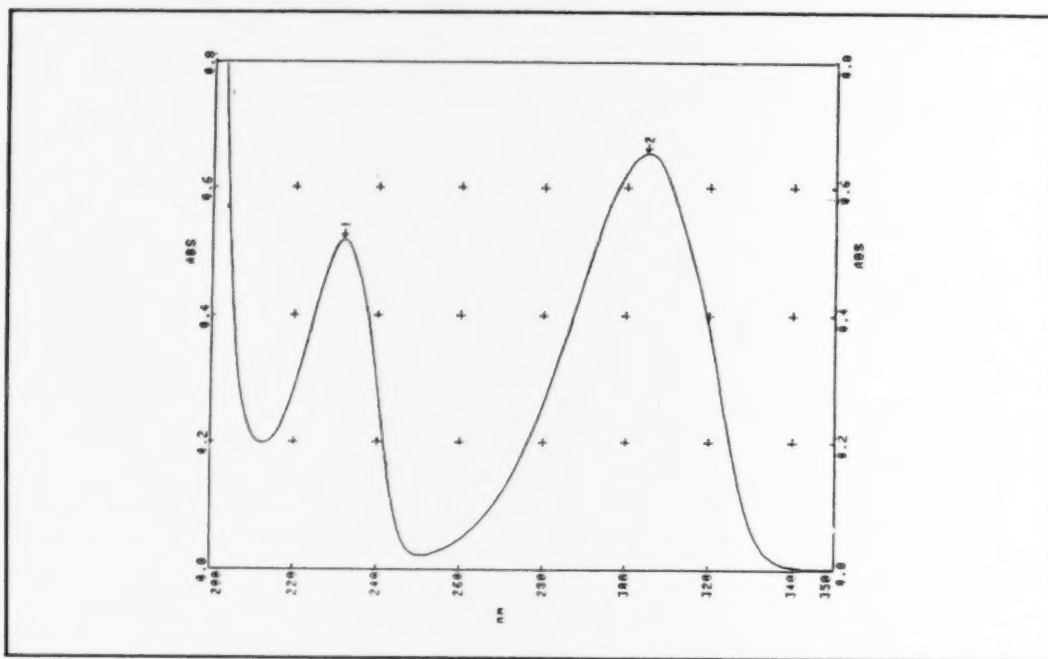


^{13}C NMR spectrum of alkaloid #5.

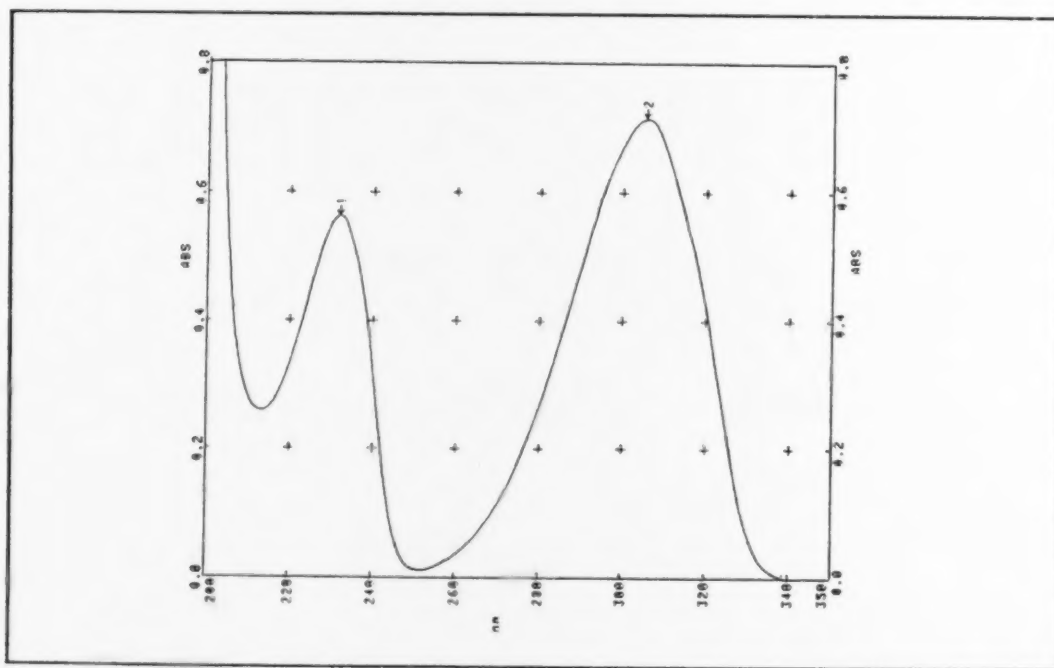


^{13}C NMR spectrum of (-)-cytisine.

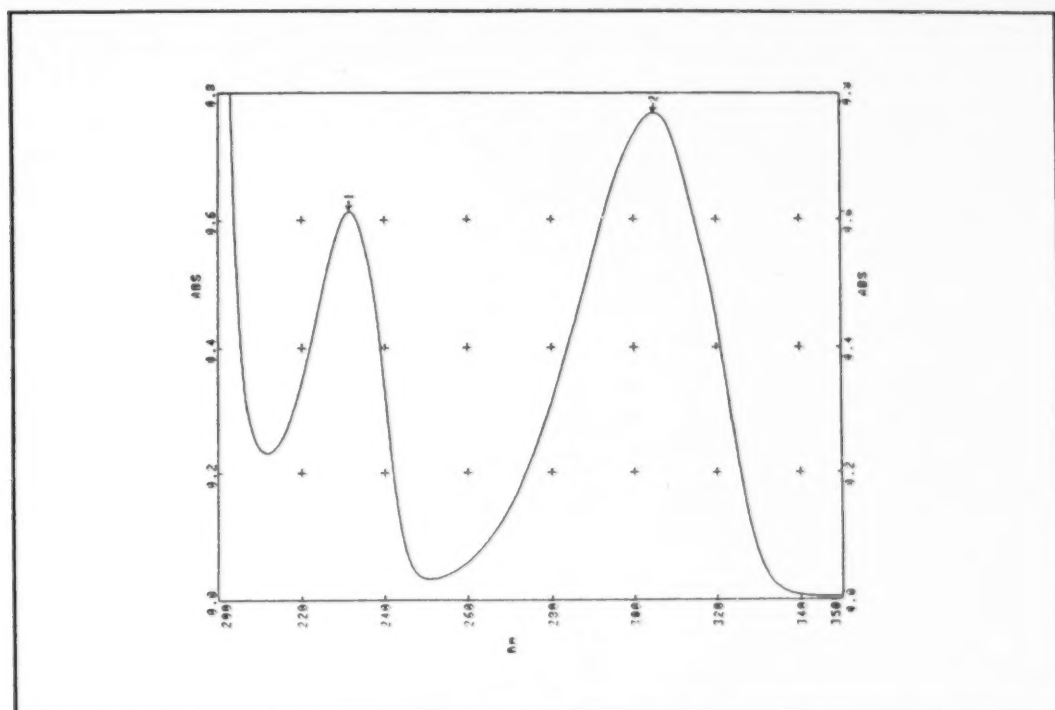
IV. UV spectra of the isolated alkaloids from Thermopsis
rhombifolia



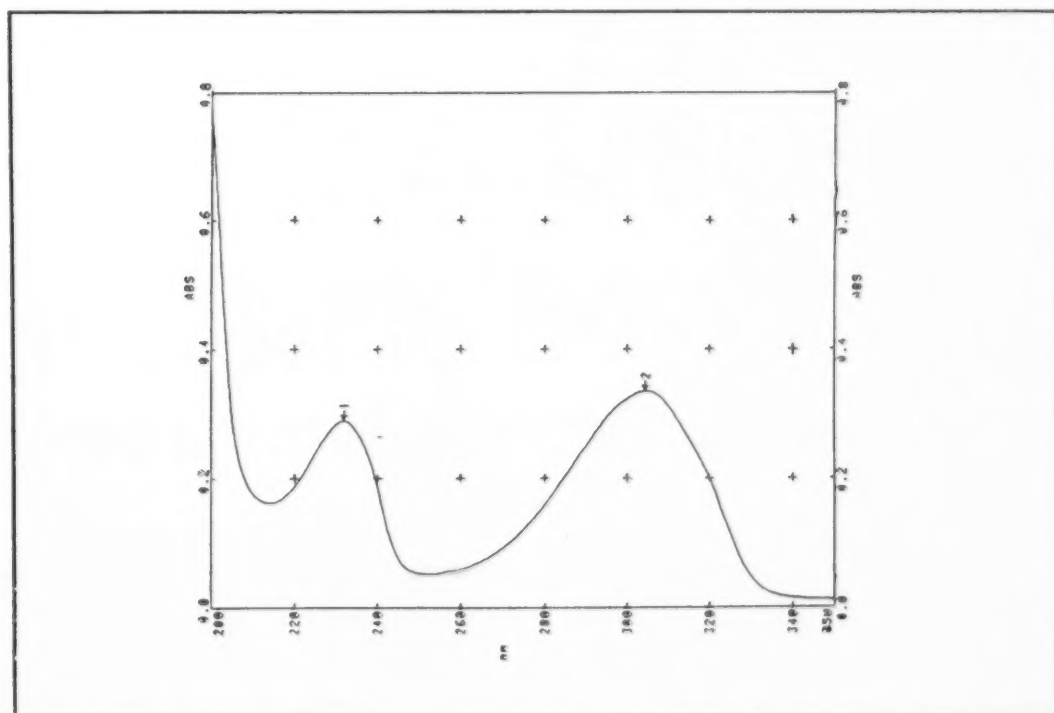
UV spectrum of alkaloid #1.



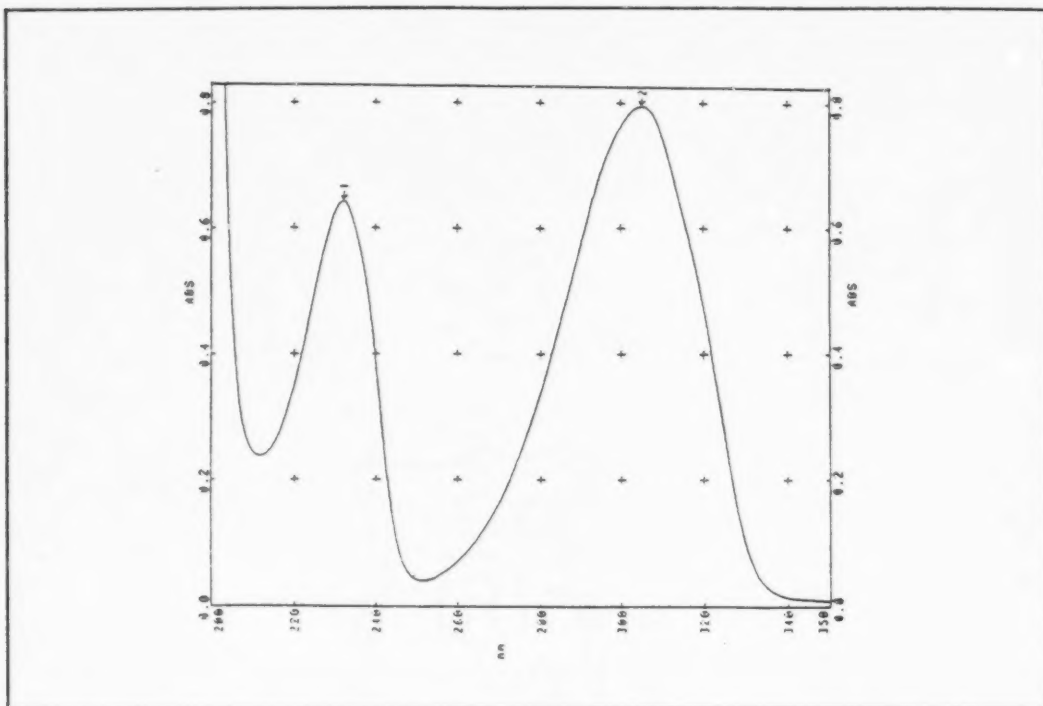
UV spectrum of alkaloid #2.



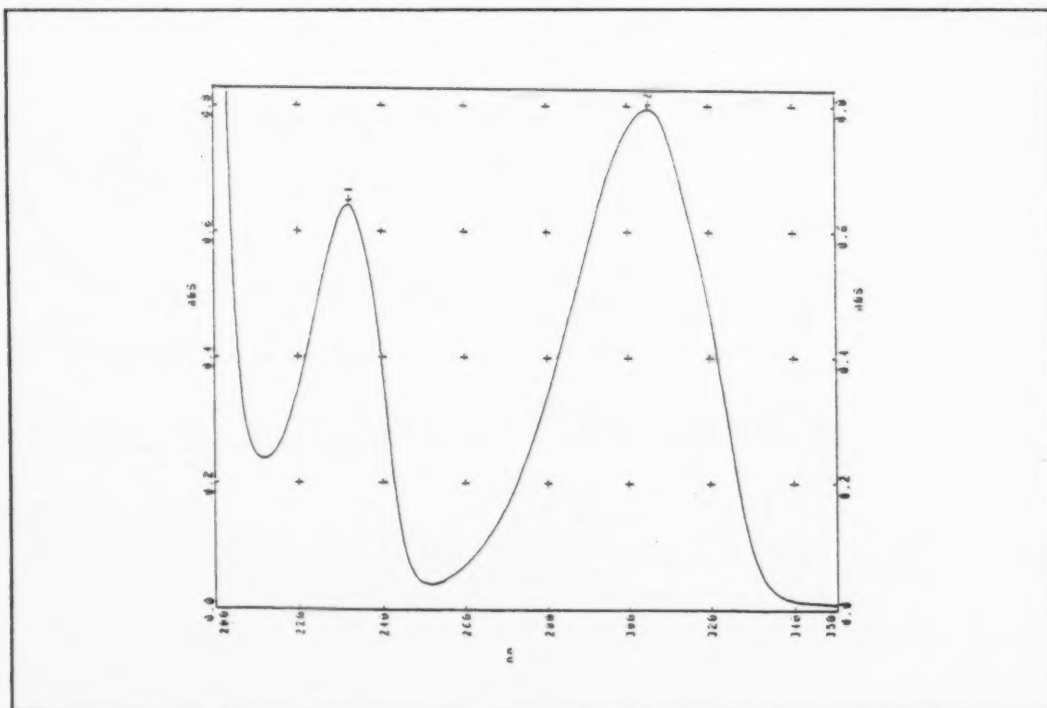
UV spectrum of alkaloid #3.



UV spectrum of alkaloid #4.



UV spectrum of alkaloid #5.



UV spectrum of (-)-cytisine.